Award Number: DAMD17-00-1-0530

TITLE: Interleukin-6 and Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Evan T. Keller, DVM, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (1 Jul 00 - 30 Jun 03)

4. TITLE AND SUBTITLE
Interleukin-6 and Prostate Cancer Progression

5. FUNDING NUMBERS
DAMD17-00-1-0530

6. AUTHOR(S)
Evan T. Keller, DVM, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, Michigan 48109-1274

E-Mail: etkeller@umich.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Prostate carcinoma (PCA) initially responds to androgen deprivation. However, it usually reoccurs in a form that is unresponsive to further hormonal manipulations. This latter form of PCA, termed androgen independent cancer, inexorably progresses resulting in the demise of the patient. Interleukin-6 (IL-6) and IL-6 receptor are expressed in PCA and activates the androgen receptor (AR). In the current work we explored the hypothesis that IL-6 contributes to the progression of PCA that is observed post-androgen deprivation, through enhancing AR activity. We determined that blocking IL-6 diminishes development and progression of androgen-independent human PCA in a murine model. Furthermore, we have identified several transcription factors that are candidate mediators of this effect. These data strongly support a role for IL-6 in the progression of prostate cancer to an androgen-independent tumor and provide potential molecular therapeutic targets.

14. SUBJECT TERMS
interleukin-6, prostate cancer, growth factor, cytokine

15. NUMBER OF PAGES
69

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....6

Reportable Outcomes.....6

Conclusions.....6

References.....7

Appendices.....8

INTRODUCTION:

Prostate carcinoma (PCA) initially responds to androgen deprivation. However, it usually reoccurs in a form that is unresponsive to further hormonal manipulations. This latter form of PCA, termed androgen independent cancer, inexorably progresses resulting in the demise of the patient. The mechanism responsible for development of androgen independent cancer is unknown. However, some clues may be found in the response of PCA cells to the cytokine interleukin-6 (IL-6) (reviewed in attached manuscript 1). Specifically, IL-6 and IL-6 receptor are expressed in PCA. Furthermore, inhibition of IL-6 in prostate cell culture diminishes PCA cell proliferation demonstrating the presence of an autocrine mechanism of IL-6 activity. Finally, IL-6 has been shown to both activate the androgen receptor (AR) in the absence of androgen and sensitize the AR to androgen. These observations have important implications regarding androgen-deprivation therapy. In the current work we explored the hypothesis that IL-6 contributes to the progression of PCA, that is observed post-androgen deprivation, through enhancing AR activity. We tested the hypothesis by the following combination of *in vitro* and *in vivo* objectives: *Objective I: Determine the mechanism through which IL-6 sensitizes AR to androgen. Objective II: Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model. Objective III: Determine if IL-6 contributes to PCA progression post-androgen deprivation* In summary, these experiments identified the extent and mechanism of IL-6's role in PCA progression. These data provide a rationale for target IL-6 for inhibiting PCA progression.

BODY:

Statement of Work Tasks for the Initial Funding Period:

Task 1. Determine the mechanism through which IL-6 sensitizes AR to androgen. (months 1-18)

- perform Western and PCR analyses to determine if IL-6 increases AR expression (months 1-3)
- perform transfection experiments to determine if IL-6 increases AR gene activation (months 4-9)
- perform transfection experiments to determine if IL-6 increases AR transactivation strength (months 10-12)
- perform bandshift assays to determine if IL-6 increases nuclear levels of AR (months 13-14)

These aims have all been accomplished (presented in attached manuscript 2). In summary:
We found that in the absence of androgen, IL-6 increased prostate-specific antigen (PSA) mRNA levels and activated several androgen-responsive promoters, but not the non-androgen responsive promoters in LNCaP cells. Bicalutamide, an antiandrogen, abolished the IL-6 effect and IL-6 could not activate the PSA and murine mammary tumor virus reporters in AR-negative DU-145 and PC3 cells. These data indicate the IL-6 induces an androgen response in CaP cells through the AR. Pretreatment of LNCaP cells with SB202190, PD98059, or tyrphostin AG879 [p38 mitogen-activated protein kinase (MAPK), MAP/extracellular signal-regulated protein kinase kinase 1/2, and ErbB2 MAPK inhibitors, respectively) but not wortmannin (PI3-kinase inhibitor) blocked IL-6-mediated induction of the PSA promoter, which demonstrates that IL-6 activity is dependent on a MAPK pathway. Finally, IL-6 activated the AR gene promoter, resulting in increased AR mRNA and protein levels in LNCaP cells. These results demonstrate that IL-6 induces AR expression and are the first report of cytokine-mediated induction of the AR promoter. Taken together, our results suggest that IL-6 induces AR activity through both increasing AR gene expression and activating the AR in the absence of androgen in CaP cells. These results provide a mechanism through which IL-6 may contribute to the development of androgen-independent CaP.

Task 2. Produce reagents needed for Task 3 (months 1-12)

- prepare anti-murine IL-6 and anti-murine isotype antibodies for Tasks 3 and 4 by inoculating mice with hybridoma, collecting ascites fluid, purifying antibodies (months 1-4)
- maintain tumor in nude mice until ready for transplantion [20 mice] (months 1-12)

We obtained the antibody from a collaborator as described in the previous progress report and used these materials as reported in manuscript (3).

Task 3. Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model [80 mice] (months 10-21)

- initiate tumor model in sham operated or orchiectomized nude mice and administer IL-6 and isotype antibody (months 10-16).
- euthanize mice, analyze tumor tissue for growth, AR/IL-6 expression and androgen sensitivity (months 17-21)

We have performed an in vivo study that demonstrates anti-IL-6 inhibits **development** of prostate tumor growth (as reported in the attached manuscript 3). In summary:
To determine if autocrine IL-6 production contributes to prostate cancer growth and chemotherapy resistance *in vivo*, xenografts of a human prostate cancer cell line that produces IL-6 (PC-3 cells) were established in nude mice then either etoposide (or saline vehicle) and anti-IL-6 monoclonal antibody (Ab) or isotype Ab were administered for 4 weeks. Anti-IL-6 Ab (with saline or etoposide) induced tumor apoptosis and regression (~60% compared to initial tumor size). Etoposide did not induce tumor regression or apoptosis in this animal model and there was no synergy between anti-IL-6 Ab and etoposide. These studies suggest that IL-6 contributes to prostate tumor growth and that targeting IL-6 may contribute to prostate cancer therapy.

We have also performed an initial experiment in which we tested the ability of blocking IL-6 (with antibody) to prevent **progression** of prostate cancer. To perform this, we established tumors in mice, then castrated the animals and initiated anti-IL-6 or isotype control antibody administration. These results are reported in an abstract that is going to be presented at the American Association of Cancer Research Annual Meeting in July, 2003 (abstract attached). In brief: xenografts of the androgen dependent human CaP line LuCAP-35 were established in nude mice. Then mice were orchiectomized and either anti-IL-6 monoclonal antibody (mAb) or isotype-control IgG antibody were immediately administered and continued for 18 weeks. Tumors were measured twice weekly. Anti-IL-6 mAb decreased tumor growth whereas the tumor continued to proliferate in mice receiving control antibody (Fig. 1). Additionally, anti-IL-6 Ab resulted in a higher survival rate compared to the control group (Fig. 1). We conclude that IL-6 is required for the transition from androgen dependent to independent development of LuCaP xenografts in nude mice.

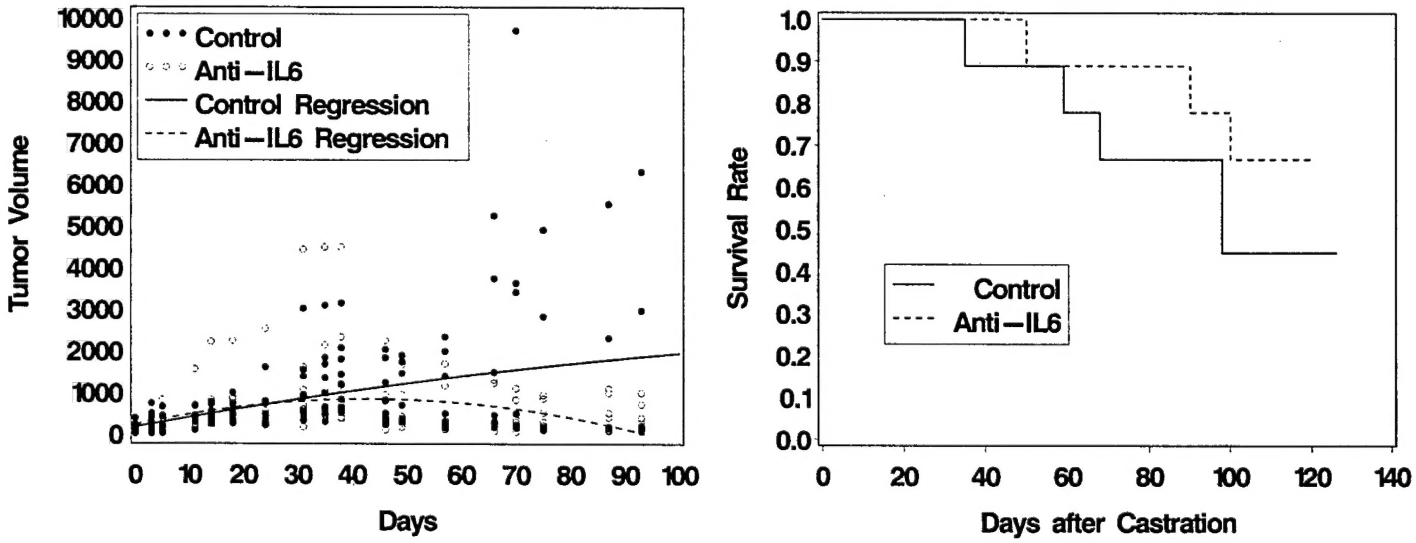

FIGURE 1. Mice were injected subcutaneously with LuCaP prostate cancer cells. Tumors were allowed to develop over a period of approximately 4 weeks, then the mice were castrated and either control IgG or anti-IL-6 antibody was administered (n=10/group). Treatment with anti-IL-6 antibody reduced tumor volume by greater than 50% and increased survival of the mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Creation of several prostate cancer cell lines that are stably transfected with androgen receptor (AR)-GFP fusion protein.
- Visualization using the AR-GFP cell lines that IL-6 induces nuclear translocation.
- Identification that several kinase inhibitors can block IL-6-mediated AR nuclear translocation.
- Completed tumor establishment and anti-IL6 experiments that demonstrate IL-6 mediates progression of established prostate tumors in a murine model.
- Performed an initial experiment to determine the role of IL-6 in tumor progression to androgen independence.
- We have identified a variety of transcription factors activated by IL-6 in prostate cancer cells using an array technology that allows us to identify transcription factors in nuclear extracts.

REPORTABLE OUTCOMES:

1. MANUSCRIPTS
    a. Smith PC and Keller ET. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. Prostate, 48:47-53, 2001.
    b. Lin DL, M Whitney, Z Yao, Keller ET. Interleukin-6 induces androgen receptor activity through up-regulation of receptor expression in prostate cancer cells. Clin Cancer Res, 7:1773-1781, 2001.
    c. Smith PC, Hobish A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine and Growth Factor Rev. 12:33-40, 2001.
    d. Evan T. Keller, Jian Zhang, Carlton R. Cooper, Peter C. Smith, Laurie K. McCauley, Kenneth J. Pienta and Russell S. Taichman. Prostate carcinoma skeletal metastases: Cross-talk between tumor and bone. Cancer Metastasis Reviews 20:333-349, 2001.
    e. Keller ET. The role of osteoclastic activity in prostate cancer skeletal metastases. Drugs Today, 38:91-102, 2002.
    f. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res, 62: 1832-1837, 2002.
2. ABSTRACTS
    a. Lauren Wallner, Peter C. Smith, Jinlu Dai, Rodney Dunn, Jian Zhang, Zhi Yao, Evan T. Keller Anti-Interleukin-6 Monoclonal Antibody Reduces Progression of Androgen Independent Human Prostate Cancer Xenografts in Nude Mice. AACR Annual Meeting, Washington DC, 2003.
    b. Smith PC, Wallner, L, Keller ET. Interleukin-6 and androgen receptor cofactors in prostate cancer xenografts and cell lines. AACR, 2002.
    c. P. C. Smith, J.L. Dai, and E.T. Keller. Unit for Laboratory Animal Medicine, Dept. of Pathology, and Institute of Gerontology, University of Michigan, Ann Arbor, MI, 48109-0940. ANTI-INTERLEUKIN-6 ANTIBODY ENHANCES CHEMOTHERAPEUTIC-MEDIATED INHIBITION OF PROSTATE CANCER CELL GROWTH IN VITRO.

CONCLUSIONS:

Our in vivo results document that IL-6 mediates a role in prostate cancer progression in vivo in an animal model. Furthermore our in vivo results demonstrate that IL-6 contributes to the progression of prostate tumors to androgen independence. These results, in combination with previously published results that IL-6 levels are elevated in men with advanced prostate cancer, strongly suggest that IL-6 is a relevant target for preventing progression of prostate cancer. Additionally, our in vitro data show that signal transduction cascades are required for IL-6 to mediate the activation of AR. The identification of signal cascades provides potential targets to block IL-6's contribution to prostate cancer.

REFERENCES:

1. Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev 2001;12:33-40.
2. Lin DL, Whitney MC, Yao Z, Keller ET. Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. Clin Cancer Res 2001;7:1773-1781.
3. Smith PC, Keller ET. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. Prostate 2001;48:47-53.

The Prostate 48:47–53 (2001)

Anti-Interleukin-6 Monoclonal Antibody Induces Regression of Human Prostate Cancer Xenografts in Nude Mice

Peter C. Smith[1] and Evan T. Keller[1,2,3,4]*

[1]Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan
[2]Department of Pathology, University of Michigan, Ann Arbor, Michigan
[3]Connective Tissue Oncology, University of Michigan, Ann Arbor, Michigan
[4]Institute of Gerontology, University of Michigan, Ann Arbor, Michigan

BACKGROUND. Despite clinical associations and in vitro data suggesting that autocrine interleukin-6 (IL-6) production contributes to prostate cancer progression or chemotherapy resistance, there have been no reports that explore the role of IL-6 on prostate tumors in vivo. In the present study, we investigated the effect of IL-6 inhibition on the growth of human prostate cancer xenografts in nude mice.

METHODS. To determine if autocrine IL-6 production contributes to prostate cancer growth and chemotherapy resistance in vivo, xenografts of a human prostate cancer cell line that produces IL-6 (PC-3) were established in nude mice. The mice were randomly divided into four treatment groups: (1) saline (vehicle control) + murine IgG (isotype control); (2) etoposide + murine IgG; (3) saline + anti-IL-6 monoclonal antibody; and (4) etoposide + anti-IL-6 monoclonal antibody. Tumors were measured twice weekly during a 4-week treatment period. At the conclusion of the study, all mice were sacrificed, and in addition to final volume, tumors were evaluated for the degree of apoptosis by TUNEL analysis.

RESULTS. Anti-IL-6 Ab (with saline or etoposide) induced tumor apoptosis and regression (~60% compared to initial tumor size). Etoposide alone did not induce tumor regression or apoptosis in this animal model, and there was no synergy between anti-IL-6 Ab and etoposide.

CONCLUSIONS. These studies suggest that IL-6 contributes to prostate cancer growth in vivo, and that targeting IL-6 may contribute to prostate cancer therapy. *Prostate 48:47–53, 2001*

KEY WORDS: **cytokine; interleukin-6; antibody; prostate cancer; chemotherapy**

INTRODUCTION

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the United States. In 1999, it was estimated that 179,300 patients were diagnosed with prostate cancer, and 37,000 patients died from the disease [1]. Radical prostatectomy can be curative in patients with localized prostate cancer. Unfortunately, many patients have an advanced form of the disease at the time of diagnosis, and require systemic androgen-ablation therapy. Initially the cancer appears as an androgen-sensitive phenotype and is responsive to this treatment. However, after a median time of 12–18 months, it commonly recurs as a hormone-refractory phenotype that is also resistant to other therapeutic

Grant sponsor: USAMRMC Prostate Cancer Research Program; Grant number: DAMD17-00-1-053; Grant sponsor: National Institutes of Health; Grant numbers: T32 RR07008 and SPORE 1 P50 CA69568.

*Correspondence to: Evan T. Keller, DVM, Ph.D, Unit for Laboratory Animal Medicine, and Department of Pathology, University of Michigan School of Medicine, 1500 E. Medical Ctr. Dr., Ann Arbor, MI 48109-0940. E-mail: etkeller@umich.edu

Received 18 December 2000; Accepted 2 March 2001

modalities including chemotherapy [2]. The precise mechanism of drug resistance in prostate cancer is not fully understood, but the secretion of protective factors by these tumors may play a role.

The cytokine interleukin-6 (IL-6) has been implicated in a number of pathophysiologic processes including stimulation of tumor proliferation [3]. In the past few years, evidence has been accumulating that IL-6 may contribute to the progression of prostate cancer [4]. For example, IL-6 serum levels are correlated with morbidity and tumor burden of prostate cancer patients [5]. Furthermore, the addition of anti-IL-6 antibody to the growth medium of androgen-independent prostate cancer cell lines has been shown to inhibit cell growth [6,7]. Additionally, inhibition of IL-6 activity enhances the cytotoxic activity of certain chemotherapeutic agents in prostate cancer cell lines that are resistant to the drugs [8]. In spite of the many studies demonstrating that IL-6 promotes prostate cancer proliferation and survival in cell culture, there has been no in vivo evidence to confirm that IL-6 contributes to prostate cancer growth. Accordingly, to determine if IL-6 contributes to prostate cancer progression, we examined the effect of inhibiting IL-6 activity on prostate cancer progression in mice implanted with a human prostate cancer xenograft.

MATERIALS AND METHODS

Cell Lines

The hormone-independent prostatic carcinoma cell lines PC-3 and DU145, and the hormone-dependent cell line, LNCaP (ATCC) were cultured in complete medium (RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin solution containing 10,000 units/ml penicillin G and 10,000 μg/ml streptomycin). All three cell lines were grown in a humidified incubator at 37°C in 5% CO_2. Cells were treated with trypsin-EDTA, washed, and resuspended in complete medium prior to their use in cytotoxicity assays.

Antibodies

Anti-hIL-6 (CLB, Amsterdam, The Netherlands) antibody is a mouse monoclonal (subtype IgG1) specific for human IL-6 [9]. Mouse $IgG1_k$ (Sigma, St. Louis, MO) was used as an isotype control antibody.

Etopsoide

Etoposide (Sigma) was dissolved in 1 ml DMSO to a final stock concentration of 25 mg/ml and stored at 4°C. The stock solution was diluted to the indicated concentrations in complete medium (in vitro experiments) or normal saline (in vivo experiments) immediately prior to use.

IL-6 Measurements in Cell Culture

Cells were grown in 10-cm polystyrene tissue culture dishes. Confluent cells were washed once with phosphate-buffered saline and then incubated for 48-hr in complete medium, at which time the supernatant was collected and stored at −20°C until assayed. IL-6 concentration was measured using a commercial ELISA kit (Quantikine Human IL-6 ELISA Kit; R&D, Systems Inc., Minneapolis, MN) as directed by the manufacturer.

In some cases, IL-6 levels were measured following the addition of anti-IL6 antibody. In these instances, cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5-ml complete medium. Anti-IL6 monoclonal antibody, isotype control (final concentration of 500 ng/ml), or complete medium was added to appropriate wells and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and stored at −20°C until ELISA was performed.

Serum IL-6 Levels

At the time of sacrifice, blood samples were collected via cardiac puncture and centrifuged at 2,700 rpm for 10 min. The serum was removed from each sample and stored at −80°C until assayed for IL-6 using the B9 cell IL-6 bioassay as previously described [10].

In Vitro Cytotoxicity Experiments

Cell lines were seeded in 96-well plates at a density of 2×10^3 cell/well in 100-μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium, and etoposide was added in a 50-μl/volume to reach a final concentration of 0.1 or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to cells that did not receive etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay (Promega, Madison, WI) as directed by the manufacturer. Cytotoxicity was calculated as follows:

$$\%\,\text{cytotoxicity} = [1 - (\text{absorbance of experimental wells}/\text{absorbance of control wells})] \times 100.$$

Mice

Eight-week-old nude (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were kept in a specific pathogen free colony, in microisolator cages, and were fed sterile rodent chow and sterile water ad libitum. All protocols were approved by the University of Michigan Animal Care and Use Committee.

In Vivo Experiments

Confluent PC-3 cells were harvested by trypsinzation, washed twice with PBS and resuspended at a density of 1×10^7 cells/ml. The mice were injected subcutaneously with 100 μl of the tumor cell suspension (10^6 cells) combined with 100 μl of Matrige® (Bectin-Dickson, Bedford, MA). The mice were monitored for tumor growth, and when tumors were detected by palpation, measurement of the tumors began. Tumor volumes were calculated by the formula: Volume = [(minimum measurement)^2 (maximum measurement)] ÷2 [as described in Ref. [11]]. Tumors were measured every other day, and when tumors reached a volume of 126 mm^3 the mice were randomly assigned to one of four treatment groups (n = 10/group). Treatment groups included isotype + saline, isotype + etoposide, anti-IL-6 + saline, and anti-IL-6 + etoposide. The treatment regimen consisted of weekly i.p. injection of anti-IL6 or isotype antibody at 500 μg/mouse/week as previously described [12] and daily i.p. injections of etoposide at 50 mg/m^2/day, which is the human-equivalent dose [13], or an equal volume of saline. Mouse IL-6 does not react with human IL-6 receptor [14]. Thus, using an anti-human-IL-6 alone will inhibit the IL-6 specifically produced by the human prostate cancer cells. Treatment continued for 4 weeks, during which time the tumors were measured on a twice-weekly basis. At the conclusion of the study all mice were sacrificed, and blood and tissue samples were collected for further analysis.

Tumor Histopathology and Detection of Apoptosis

Excised tumors were placed in 10% formalin, embedded in paraffin and sectioned at 10 μM thickness. Sections were examined utilizing standard hematoxylin and eosin (H&E) staining for routine histopathology. To evaluate apoptosis, sections were deparaffinized, rehydrated, and subjected to terminal deoxytransferase UTP end-labeling (TUNEL) analysis using ApopTag® Plus Peroxidase Kit (Intergen, Purchase, NY) according to the manufacturer's directions. The number of apoptotic nuclei per 200X field (averaged from three random 200X fields) was determined for each section by an investigator that was blinded to the samples as previously described [15].

Statistical Analysis

To determine differences among treatment groups for tumor size, two-way analysis of variance (ANOVA) was used followed by Fisher's least significant difference for post-hoc analysis. Statistical significance was determined at $P < 0.05$.

RESULTS

IL-6 Secretion by Prostate Cancer Cell Lines

The detection of IL-6 secretion by prostate cancer cell lines is fairly inconsistent between laboratories [6,7,16]. Thus, it was critical to determine the IL-6 expression of various cell lines in our laboratory prior to proceeding with an in vivo challenge. Accordingly, we measured the amount of IL-6 secreted into the culture supernatant of various prostate cancer cell lines. PC-3 cells secreted the most IL-6 followed by DU-145 cells (Fig. 1A). IL-6 was undetectable in LNCaP cell culture supernatant (Fig. 1A).

Effect of IL-6 Antibody Treatment on IL-6 Levels in PC-3 Cell Culture Supernatant

In order to provide a maximum challenge to our ability to inhibit IL-6, we performed the remaining experiments with the PC-3 cells, which secreted the highest levels of IL-6. To confirm that the anti-IL-6 antibody we were using effectively inhibited IL-6 levels over a length of time, we incubated PC-3 cells with 500 ng/ml of either anti-IL-6 or isotype control antibody for 24, 48, and 72 hr, then measured IL-6 levels using ELISA. Anti-IL-6 antibody decreased the detection of IL-6 by ≥50% at all three time points compared to the isotype antibody (Fig. 1B).

Effect of Anti-IL-6 and Etoposide on Cell Proliferation of PC-3 Cells

To determine if anti-IL-6 antibody enhances the etoposide-mediated cytotoxicity of prostate cancer cells, we incubated prostate cancer cells with antibody and etoposide, then measured viable cell number. Anti-IL-6 antibody alone decreased the number of viable cells by approximately 10% (Fig. 2). Etoposide alone at low (0.1 μM) and high (10 μM) doses induced approximately 5% cytotoxicity. Anti-IL-6 antibody combined with the high dose of etoposide induced approximately 25% cytotoxicity, thus demonstrating a synergistic effect between etoposide and anti-IL-6 antibody in vivo. These in vitro data provided the rationale to pursue the ability of IL-6 antibody to modulate prostate cancer cell growth in vivo.

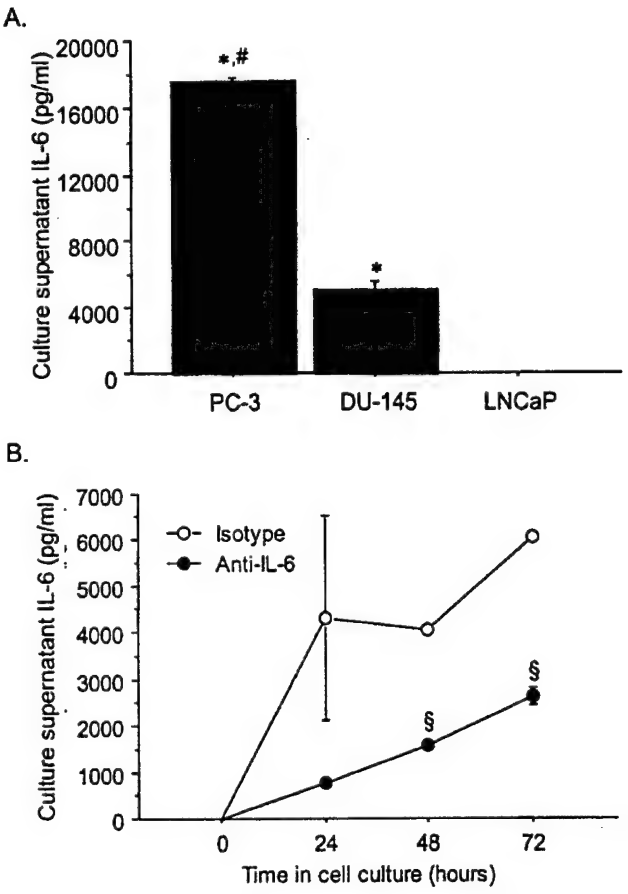

Fig. 1. Anti IL-6 antibody diminished ELISA detectable IL-6 from PC-3 cells. **A:** The indicated prostate cancer cell lines were plated at a density of 5×10^6 cells/10 ml in 10 cm tissue culture plates and cultured for 48 hr. Supernatant was then collected and subjected to ELISA for IL-6. **B:** PC-3 cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5 ml complete medium. Anti-IL-6 monoclonal antibody or isotype control (final concentration of 500 ng/ml) was added and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and subjected to ELISA for IL-6. All time points were run on the same plate. Both assays were performed in triplicate. $^*P < 0.01$ vs. LNCaP, $^{\#}P < 0.01$ vs. DU-145, $^{\S}P < 0.05$ vs. isotype.

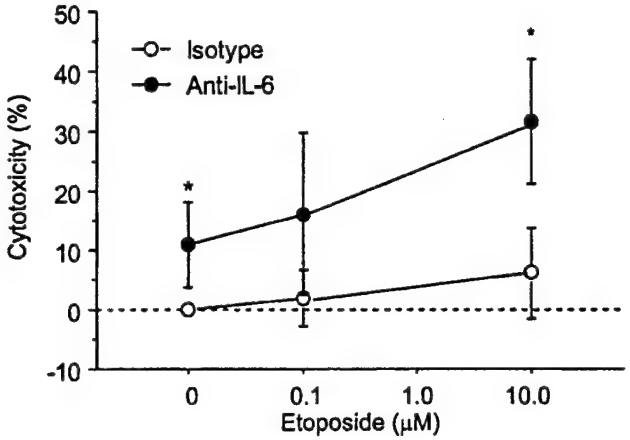

Fig. 2. Inhibition of IL-6 induces cytotoxicity of PC-3 cells in vitro. Cell lines were seeded in 96-well plates at a density of 2×10^3 cells/well in 100 μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium. Etoposide was added in a 50 μl/volume to reach a final concentration of 0.1 μg/ml or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to wells not receiving etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay and cytotoxicity was determined as described in the Methods section. The assay was performed in triplicate $^*P < 0.05$ vs. isotype.

Tumor Response

Based on our observation that inhibition of IL-6 alone inhibited PC-3 survival in addition to enhancing-mediated cytotoxicity in vivo, we next explored if inhibiting IL-6 would mimic these effects in vivo on established prostate cancer tumors. To accomplish this, PC-3 cells were subcutaneously injected into nude mice. The tumors were allowed to develop until they were approximately 126 mm^3, at which time anti-IL-6 or isotype antibody and etoposide administration was initiated. Antibody was administered at a level that inhibited IL-6 bioactivity by approximately 20% (based on B9 bioassay; data not shown). Treatment was continued for a 4-week period. Using an ELISA specific for human IL-6 (and non-cross-reactive with murine IL-6), we determined that serum human IL-6 levels were 30.1 ± 10.4 pg/ml in tumor-implanted saline + isotype control mice, compared to undetectable levels in mice not implanted with tumor, demonstrating that the tumors produced IL-6 in vivo. The tumors in the isotype-treated mice had a continuous, albeit slow, tumor growth rate, whereas the tumors in the istotype + etoposide-treated mice did not grow (Fig. 3). In contrast, the final tumor volumes were reduced by approximately 60% compared to their initial size in the mice receiving anti-IL-6 or anti-IL6 + etoposide. Furthermore, they were approximately 75% smaller than the tumors in the mice receiving isotype alone (Fig. 3). There was no significant difference between the tumor volumes in the mice treated with istotype alone compared to the mice treated with etoposide alone.

Apoptosis

We evaluated the effect of anti-IL-6 antibody and etoposide on the amount of apoptosis present in the PC-3 tumors. Routine histological evaluation of the tumors did not demonstrate any differences among the treatment groups. However, administration of anti-IL-6 antibody was associated with marked apop-

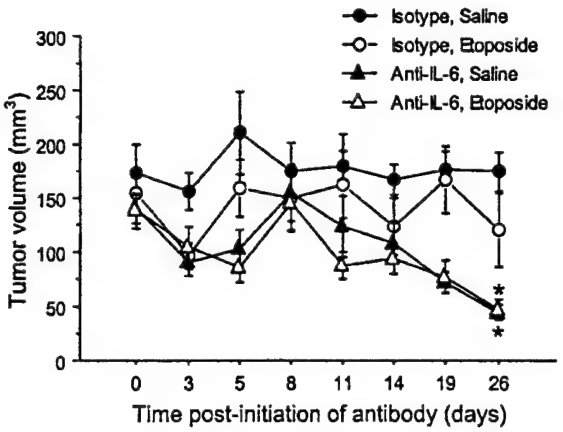

Fig. 3. Inhibition of IL-6 induces PC-3 xenograft regression in mice. PC-3 cells (10^6) in a Matrigel slurry were subcutaneously implanted in male nu/nu mice. When tumors reached 126 cm^3, weekly i.p. injections of anti-IL6 or isotype antibody (500 μg/mouse) and daily i.p. injections of etoposide (50 mg/m^2) or saline vehicle were initiated. Tumors were measured twice-weekly for 4 weeks. There were 10 animals/group. Results are shown as mean ± SD. *$P < 0.01$.

tosis in the tumors from both the saline vehicle and etoposide treated mice, compared to the moderate level of apoptosis in the tumors from the isotype and isotype + etoposide-treated mice (Fig. 4). These data demonstrate that IL-6 has an anti-apoptotic action in PC-3 cells in vivo.

DISCUSSION

A large body of evidence has accumulated that suggests IL-6 contributes to prostate cancer progression. The evidence includes both clinical observations that increased levels of IL-6 are associated with increasing grade of prostate cancer in patients [5,17,18] and in vitro experiments that have demonstrated that IL-6 promotes prostate cancer cell growth and prevents chemotherapeutic-mediated cytotoxicity [7,19]. In the current study, we provide the first demonstration that IL-6 activity promotes prostate cancer growth in vivo.

Our data demonstrate that high levels of IL-6 are secreted by PC-3 and DU-145 cells, whereas IL-6 levels were not detectable using ELISA methodology in LNCaP cells. These findings are consistent with previous reports on IL-6 secretion by prostate cancer cell lines [6,8]. PC-3 and DU-145 cells are androgen non-responsive, whereas LNCaP cells are androgen-responsive. Thus, these results suggest that loss of androgen responsiveness is associated with increased IL-6 expression. This postulation is consistent with the observations that elevation of serum IL-6 levels is associated with increasing grade of prostate cancer [5,18,20]. A cause and effect cannot be determined based on the current data. However, it has been previously reported that the androgen dihydrotestosterone inhibits IL-6 expression in prostate cancer cells [16] and that orchiectomy increased IL-6 expression in murine bone marrow [10]. Thus, it is plausible that loss of androgen-response promotes IL-6 expression.

Prostate cancer is poorly responsive to chemotherapy. Therefore, a mechanism to enhance chemotherapeutic killing of tumors would be a boon for prostate cancer patients. Borsellino et al. [7] have reported that inhibition of IL-6 activity enhances chemotherapeutic killing of prostate cancer cell in vitro. However, this effect has not been reported in vivo. In the current study, the cell type and therapeutic agent were chosen based on in vitro studies, and the human equivalent dose of etoposide was used to calculate the dosage administered to the mice. However, we did not observe an effect of etoposide on the PC-3 in vivo. In contrast, tumors responded to anti-IL-6 antibody, although the combination of anti-IL-6 and etoposide did not significantly enhance this inhibitory effect compared to anti-6 alone. Taken together, these data suggest that inhibition of IL-6 does not enhance etoposide-mediated killing in vivo. However, they clearly demonstrate that inhibition of IL-6 alone, or in the presence of etoposide, induces regression of PC-3 tumors. This observation provides in vivo evidence that IL-6 contributes to prostate cancer cell growth, and that inhibition of its activity promotes tumor regression.

This ability to inhibit IL-6 activity in humans has been previously demonstrated in several clinical trials using murine monoclonal antibodies in patients with multiple myeloma [21,22]. Early trials demonstrated the feasibility of blocking IL-6 activity in this manner, and that such therapy had beneficial effects [21]. These trials also revealed certain limitations to anti-IL-6 therapy using murine monoclonal antibodies. One such limitation is that in some patients with advanced disease, IL-6 levels were so high that the antibody was unable to neutralize them [22]. Another limitation is that development of antibodies to mouse immunoglobulin may result in rapid clearance of the murine monoclonal antibody and diminished efficacy of treatment [23]. This problem has been addressed through the chimerization [23,24] and humanization [24] of murine anti-IL-6 antibodies. Tsunenari et al. [24] demonstrated reduced antigenicity of chimeric antibodies and even lower antigenicity of humanized murine antibodies (24) while a later study by van Zaanen et al. [23] showed no induction of human anti-chimeric antibodies in multiple myeloma patients receiving chimeric anti-IL-6 antibodies. Overall, these studies suggest that inhibition of IL-6 activity in prostate cancer patients is achievable.

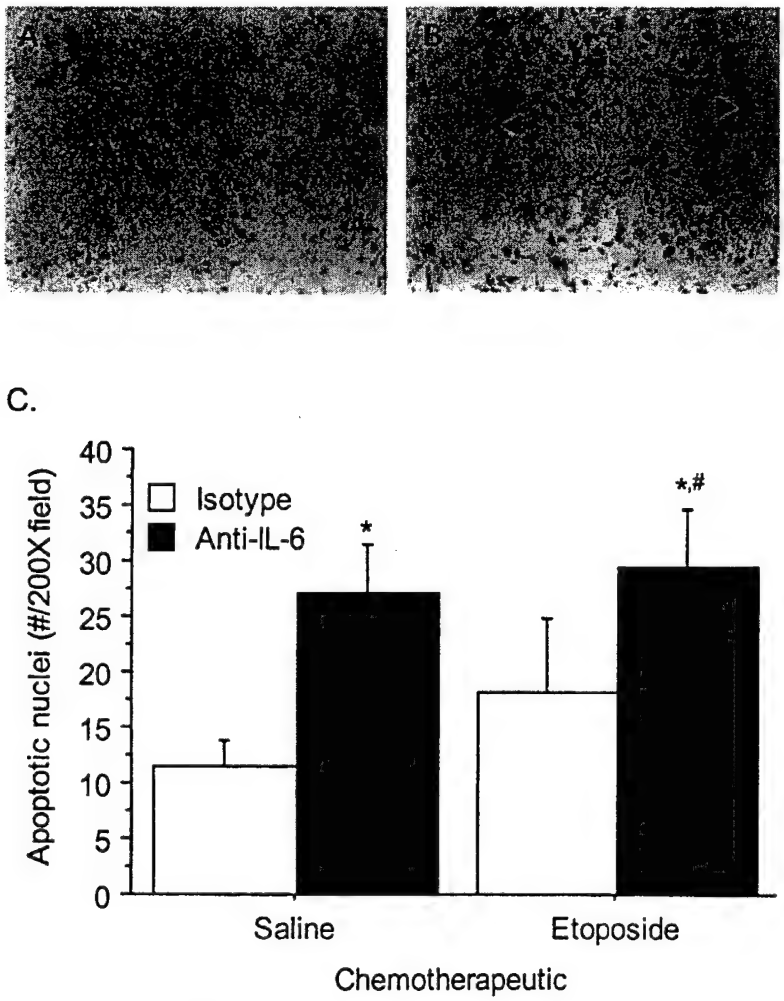

Fig. 4. Inhibition of IL-6 induces apoptosis in PC-3 tumors in mice. Tumors were excised from mice 4 weeks after initiation of anti-IL6 and etoposide as described in Fig. 3. Breaks in DNA were determined by labeling 3′OH termini using terminal deoxytransferase and staining with peroxidase. Tumor sections are shown from (**A**) isotype-treated mice and (**B**) anti-IL-6 treated mice. Apoptotic nuclei are dark brown (arrowheads). Original magnification 100 ×. **C**: To determine the degree of apoptosis, the number of apoptotic nuclei/200 × field (average of triplicate) were determined by an investigator blinded to the samples. *$P < 0.01$ compared to the saline + isotype mice; *$P < 0.05$ compared to the etoposide + isotype mice. Data are shown as mean ± SD of 4 mice/group.

The mechanism through which IL-6 contributes to overall prostate tumor growth is not clear. There are conflicting reports regarding the effect of IL-6 on prostate cancer cell proliferation in vitro [4]. Thus, it is unclear if IL-6 directly contribute to tumor growth through stimulation of cell proliferation. In addition to increased cell proliferation, a tumor may enlarge due to decreased apoptotic death of cell. The observation that IL-6 has been demonstrated to have anti-apoptotic action in several cell types [19,25,26] including the prostate cancer cell lines LNCaP and PC-3 (27) led us to evaluate IL-6's effect on apoptosis in the prostate cancer xenografts. Our observation that the level of apoptosis in tumors of mice that received anti-IL-6 compared to those who received isotype control antibody demonstrates that IL-6 protects prostate cancer cells from apoptosis in vivo. These findings are consistent with the in vitro results of Chung et al. [27] who demonstrated the antiapoptotic effects of IL-6 in PC-3 and LNCaP cell lines, and showed that this effect is the result of IL-6 activation of phosphatidylinositol (PI)-3 kinase. These previous reports, taken together with the currently reported murine studies, suggest that inhibition of apoptosis is one mechanism through which IL-6 contributes to prostate cancer progression.

CONCLUSION

In summary, the current study demonstrates that anti-IL-6 antibody induces apoptosis and regression of established PC-3 tumors in mice. However, the in vivo

data do not support the in vitro observations that IL-6 enhances etoposide-mediated killing. These data, combined with the clinical reports that IL-6 is associated with prostate cancer stage [17,18,28], provide compelling evidence that IL-6 contributes to prostate cancer progression and suggests that targeting IL-6 may induce prostate cancer regression.

REFERENCES

1. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J Clin 1999;49(1):1,8–31.
2. Mahler C, Denis L. Management of relapsing disease in prostate cancer. Cancer 1992;70(1 Suppl):329–334.
3. Keller ET, Wanagat J, Ershler WB. Molecular and cellular biology of interleukin-6 and its receptor. Front Biosci 1996;1:340–357.
4. Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev 2001;12(1):33–40
5. Twillie DA, Eisenberger MA, Carducci MA, Hseih WS, Kim WY, Simons JW. Interleukin-6: A candidate mediator of human prostate cancer morbidity. Urology 1995;45(3):542–549.
6. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role of IL-6 in the progression of prostate cancer. Prostate 1999;38(3):199–207.
7. Borsellino N, Belldegrun A, Bonavida B. Endogenous interleukin 6 is a resistance factor for *cis*-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. Cancer Res 1995;55(20):4633–4639.
8. Borsellino N, Bonavida B, Ciliberto G, Toniatti G, Travali S, D'Alessandro N. Blocking signaling through the Gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumor cells to etoposide and cisplatin-mediated cytotoxicity. Cancer 1999;85(1):134–144.
9. Brakenhoff JP, Hart M, De Groot ER, Di Padova F, Aarden LA. Structure-function analysis of human IL-6. Epitope mapping of neutralizing monoclonal antibodies with amino- and carboxyl-terminal deletion mutants. J Immunol 1990;145(2):561–568.
10. Zhang J, Pugh TD, Stebler B, Ershler WB, Keller ET. Orchiectomy increases bone marrow interleukin-6 levels in mice. Calcif Tissue Int 1998;62(3):219–226.
11. Davol PA, Frackelton AR, Jr. Targeting human prostatic carcinoma through basic fibroblast growth factor receptors in an animal model: characterizing and circumventing mechanisms of tumor resistance. Prostate 1999;40(3):178–191.
12. Finck BK, Chan B, Wofsy D. Interleukin 6 promotes murine lupus in NZB/NZW F1 mice. J Clin Invest 1994;94(2):585–591.
13. Pienta KJ, Lehr JE. Inhibition of prostate cancer growth by estramustine and etoposide: Evidence for interaction at the nuclear matrix. J Urol 1993;149(6):1622–1625.
14. Leebeek FW, Fowlkes DM. Construction and functional analysis of hybrid interleukin-6 variants. Characterization of the role of C-terminus for species specificity. FEBS Lett 1992;306(2–3): 262–264.
15. Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR. Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. Cancer Res 2000;60(15):4016–4020.
16. Keller ET, Chang C, Ershler WB. Inhibition of NFkappaB activity through maintenance of IkBa levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J Biol Chem 1996;271(42):26267–26275.
17. Akimoto S, Okumura A, Fuse H. Relationship between serum levels of interleukin-6, tumor necrosis factor-alpha and bone turnover markers in prostate cancer patients. Endocrind J 1998; 45(2):183–189.
18. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC. Elevated levels of circulating interleukin-6 and transforming growth factor-betal in patients with metastatic prostatic carcinoma. J Urol 1999;161(1):182–187.
19. Chen RH, Chang MC, Su YH, Tsai YT, Kuo ML. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J Biol Chem 1999;274(33):23013–23019.
20. Drachenberg DE, Elgamal AA, Rowbotham R, Peterson M, Murphy GP. Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. Prostate 1999;41(2):127–133.
21. Klein B, Wijdenes J, Zhang XG, Jourdan M, Boiron JM, Brochier J, Liantard J, Merlin M, Clement C, Morel-Fournier B. Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukemia. Blood 1991;78(5):1198–1204.
22. Bataille R, Barlogie B, Lu ZY, Rossi JF, Lavabre-Bertrand T, Beck T, Wijdenes J, Brochier J, Klein B. Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. Blood 1995;86(2): 685–691.
23. van Zaanen HC, Lokhorst HM, Aarden LA, Rensink HJ, Warnaar SO, van der Lelie J, van Oers MH. Chimaeric anti-interleukin 6 monoclonal antibodies in the treatment of advanced multiple myeloma: A phase I dose-escalating study. Br J Haematol 1998; 102(3):783–790.
24. Tsunenari T, Akamatsu K, Kaiho S, Sato K, Tsuchiya M, Koishihara Y, Kishimoto T, Ohsugi Y. Therapeutic potential of humanized anti-interleukin-6 receptor antibody: antitumor activity in xenograft model of multiple myeloma. Anticancer Res 1996;16(5A):2537–2544.
25. Dedoussis GV, Mouzaki A, Theodoropoulou M, Menounos P, Kyrtsonis MC, Karameris A, Maniatis A. Endogenous interleukin 6 conveys resistance to cis-diamminedichloroplatinum-mediated apoptosis of the K562 human leukemic cell line. Exp Cell Res 1999;249(2):269–278.
26. Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, Schutz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschblach F, Schurmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 *trans* signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: Evidence in Crohn disease and experimental colitis in vivo. Nat Med 2000;6(5):583–588.
27. Chung TD, Yu JJ, Kong TA, Spiotto MT, Lin JM. Interleukin-6 activates phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. Prostate 2000;42(1): 1–7.
28. Hobisch A, Rogatsch H, Hittmair A, Fuchs D, Bartsch G, Jr., Klocker H, Bartsch G, Culig Z. Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. J Pathol 2000;191(3):239–244.

Interleukin-6 Induces Androgen Responsiveness in Prostate Cancer Cells through Up-Regulation of Androgen Receptor Expression[1]

Din-Lii Lin, Mary C. Whitney, Zhi Yao, and Evan T. Keller[2]

Unit for Laboratory Animal Medicine [D-L. L., M. C. W., E. T. K.], Department of Pathology [E. T. K.], Program in Cellular and Molecular Biology [E. T. K.], and Institute of Gerontology [E. T. K.], University of Michigan, Ann Arbor, Michigan 48109, and Department of Immunology, Tianjin Medical University, Tianjin, China 300203 [Z.Y.]

ABSTRACT

Interleukin-6 (IL-6) induces prostate cancer (CaP) cell proliferation *in vitro*. Several lines of evidence suggest that IL-6 may promote CaP progression through induction of an androgen response. In this work, we explored whether IL-6 induces androgen responsiveness through modulation of androgen receptor (AR) expression. We found that in the absence of androgen, IL-6 increased prostate-specific antigen (PSA) mRNA levels and activated several androgen-responsive promoters, but not the non-androgen responsive promoters in LNCaP cells. Bicalutamide, an antiandrogen, abolished the IL-6 effect and IL-6 could not activate the PSA and murine mammary tumor virus reporters in AR-negative DU-145 and PC3 cells. These data indicate the IL-6 induces an androgen response in CaP cells through the AR. Pretreatment of LNCaP cells with SB202190, PD98059, or tyrphostin AG879 [p38 mitogen-activated protein kinase (MAPK), MAP/extracellular signal-regulated protein kinase kinase 1/2, and ErbB2 MAPK inhibitors, respectively) but not wortmannin (PI3-kinase inhibitor) blocked IL-6-mediated induction of the PSA promoter, which demonstrates that IL-6 activity is dependent on a MAPK pathway. Finally, IL-6 activated the *AR* gene promoter, resulting in increased AR mRNA and protein levels in LNCaP cells. These results demonstrate that IL-6 induces AR expression and are the first report of cytokine-mediated induction of the *AR* promoter. Taken together, our results suggest that IL-6 induces AR activity through both increasing AR gene expression and activating the AR in the absence of androgen in CaP cells. These results provide a mechanism through which IL-6 may contribute to the development of androgen-independent CaP.

INTRODUCTION

When initially identified, most CaP[3] require androgenic stimulation for growth. After androgen withdrawal, most prostate cells undergo an active process of programmed cell death (1). Unfortunately, after an initial response to androgen deprivation therapy, CaP usually recurs in a form that grows independent of androgen and is unresponsive to further androgen withdrawal (2). The mechanism responsible for development of androgen independent cancer is unknown. However, accumulating evidence suggests that the androgen-independent phenotype results when CaP cells acquire a paracrine or autocrine growth mechanism through production of growth factors and cytokines (3–5).

A putative growth factor that promotes prostate cancer growth is IL-6. Initially identified as a cytokine that exhibits pleiotropic functions, IL-6 regulates gene expression in a number of different organs, modulates immune function, stimulates the hypothalamic-pituitary axis, promotes osteoclast resorption of bone, and stimulates bone marrow (reviewed in Ref. 6). It has been shown to induce acute-phase proteins and a number of immediate early genes, including *jun B*, circulating intercellular adhesion molecule 1 (*ICAM-1*), and IFN regulatory factor 1 (*IRF1*; Refs. 7–9). The biological activities of IL-6 are mediated by the IL-6 receptor, which binds IL-6 specifically and with low affinity, and gp130, which associates with the IL-6-IL-6 receptor complex, resulting in high-affinity binding and activation of intracellular signaling. Evidence has accumulated that suggests IL-6 may be an important autocrine and/or paracrine growth factor for CaP (4, 5, 10, 11).

AR, an essential mediator of androgen action, is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (12). The receptor contains a ligand (androgen)-binding domain and a DNA-binding domain. In the absence of androgens, the AR stays mainly in the cytoplasm in an inactive form. When the AR is activated by androgen, it binds to an enhancer ARE in the regulatory region of target genes as the key step for promoter activation (13). During the progression of CaP, AR expression becomes heterogeneous (14).

Received 11/29/00; revised 3/21/01; accepted 3/26/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by US Army Medical Research Materiel Command AMRMC Prostate Cancer Research Program Grant DAMD17-00-1-053 and NIH Grant SPORE 1 P50 CA69568.

[2] To whom requests for reprints should be addressed, at Room 5304, CCGCB, 1500 East Medical Center Drive, University of Michigan, Ann Arbor, MI, 48109. Phone: (734) 615-0280; Fax: (734) 936-9220; E-mail: etkeller@umich.edu.

[3] The abbreviations used are: CaP, prostate carcinoma(s); AR, androgen receptor; ARE, androgen responsive element; BIC, bicalutamide; DHT, dihydrotestosterone; ER, estrogen receptor; IL-6, interleukin-6; lux, luciferase; MMTV, murine mammary tumor virus; NFDM, nonfat dried milk; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; SLP, sex-limited protein; PI, phosphatidylinositol; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; EGF, epidermal growth factor; GFP, green fluorescent protein; STAT, signal transducer and activator of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

LNCaP is an androgen-responsive human CaP cell line, derived from a lymph node CaP metastasis (15). LNCaP cells have AR mutations and have been shown to be sensitive to antiandrogens and estrogen as well as to androgens (16, 17). IL-6 has been shown to confer androgen-like activity on LNCaP cells (18, 19). Although the ability of IL-6 to induced androgen-like activity has been associated with activation of several signal transduction cascades (19, 20), the mechanism through which IL-6 induces AR activity is unknown. Accordingly, in the current report, we explore the mechanism through which IL-6 induces an androgen response in prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Reagents. LNCaP, PC-3, and DU-145 prostate cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). These cell lines have been previously shown by several investigators to express both the IL-6 receptor α and the IL-6 receptor β (gp130) chains (4, 5, 21). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 incubator. Recombinant human IL-6 was obtained from Sigma Chemical Co., Inc. (St. Louis, MO). Rabbit antihuman AR polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). The antiandrogen BIC (Casodex, 20 μM; Zeneca Pharmaceuticals; Macclesfield, United Kingdom) was provided by Dr. K. Olsen (University of Michigan, Ann Arbor, MI). The following reagents: BSB202190 (5 μM), PD98059 (15 μM), wortmannin (100 nM), and tyrphostin AG879 (10 μM) were purchased from Calbiochem, San Diego, CA. The PSA reporter, p1.5kPSA-Lux, containing the proximal −1500-bp of the PSA promoter driving the lux cDNA was a gift from Dr. P. Hsiao (NIEAS, Durham, NC). The MMTV (22) reporter, pMMTV-Lux, containing 150 bp of the MMTV promoter driving the lux cDNA has been described previously (23). The mouse SLP (24) reporter, pc'Δ9(slp)-Lux, containing 900 bp of the SLP promoter driving the lux cDNA is a gift from Dr. D. Robins (University of Michigan, Ann Arbor). The plasmid pPAI(−800/+22)-Lux containing a human type-1 plasminogen activator inhibitor gene promoter driving the lux cDNA was a gift from Dr. T. Gelehrter (University of Michigan, Ann Arbor). The plasmid ERE-tk-Lux, containing tandem ER response elements upstream of the thymidine kinase promoter driving the lux cDNA, was a gift from Dr. R. Koenig (University of Michigan, Ann Arbor).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as previously described, with minor modification (18). Briefly, total RNA was isolated from cells using TRIzol method (Life Technologies, Inc.). Using Superscript one-step RT-PCR system (Life Technologies, Inc.), we subjected 1 μg of total RNA to thermal cycling as follows: one cycle at 50°C for 30 min; at 94°C for 2 min with an additional 29 cycles at 94°C for 15 s; at 56°C for 30 s; and at 72°C for 1 min, and 5 min at 72°C for the final extension. PSA and β2-microglobulin primer sequences were: PSA 418/21 sense, 5'-GGCAGGTGCTTGTAGCCTCTC-3'; PSA 939/21 antisense, 5'-CACCCGAGCAGGTGCTTTTGC-3'; β2-microglobulin, sense, 5'-ATGCCTGCCGTGTGAACCATGT-3'; and β2-microglobulin antisense 5'-AGAGCTACCTGTGGAGCAACCT-3' as previously described (18). The AR primers were designed to flank the ligand-binding domain: sense, 5'-ACACATTGAAGGCTATGAATGTC-3'; and antisense, 5'-TCACTGGGTGTGGAAATAGATGGG-3'. For quantitation, either PSA or AR primers (10 μM, 1 μl) was mixed with β2-microglobulin (5 μM, 1 μl) for RT-PCR. The PCR products were then resolved in the 1.3% agarose gel and bands were analyzed with ChemiImager v3.3 software (Alpha Innotech, San Leandro, CA). Target fragment levels were normalized against β2-microglobulin, and data are presented as target mRNA:β2-microglobulin ratio.

Transient Transfection. Transfection was conducted using SuperFect (Qiagen, Valencia, CA) as recommended by the manufacturer. Briefly, LNCaP cells were plated in 6-well plates at a confluency of 60–70% 24 h before transfection in medium supplemented with fetal bovine serum or in medium supplemented with charcoal-stripped serum as indicated (See Fig. 1). Cells in 1 ml of medium in 6-well plates was incubated at 37°C with 2 μg of plasmid DNA (1 μg of reporter, internal control 50 ng of pRL-cytomegalovirus, and 950 ng pBluescript) mixed in 10 μl of SuperFect. After the 2 h incubation, 1 ml of fresh medium was added to the cells. The medium was replaced the next day, and the cells were incubated for an additional 24 h. Total protein was then collected by lysis buffer and lux activities were measured by using Dual-Luciferase System (Promega, Madison, WI) and captured by TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

Nuclear Lysate Preparation. Nuclear protein extract from LNCaP cells was prepared as described previously (23). Briefly, cells were harvested after being washed twice in PBS buffer. For nuclei preparation, cells were resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3000 × *g* centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in the lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was measured by Bradford assays.

Western Blot Analysis. LNCaP cells were cultured for 2 days in media with charcoal stripped serum. The cells were then stimulated for 24 h with the indicated concentrations of IL-6 and then lysed by multiple freeze thawing in 0.25 M Tris buffer. Western blot analysis was performed as previously described (23) with rabbit anti-AR (N-20, Santa Cruz Biotechnology).

Detection of AR by Indirect Immunofluorescence and Fluorescence Imaging. LNCaP cells were grown on glass coverslips for immunofluorescence. The coverslips were rinse once in PBS and fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min. Neutralization took place for 5 min in 50 mM NH_4Cl in PBS. The coverslips were then washed twice in PBS. Cells were incubated for 15 min with 1% BSA, NFDM, 0.3% Triton X-100 in PBS. The coverslips were incubated for 1 h in rabbit anti-AR (N-20) diluted 1:100 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The slips were then extensively washed in PBS-Tween-20 and incubated for 1 h with goat antirabbit IgG conjugated with FITC (Santa Cruz Biotechnolo-

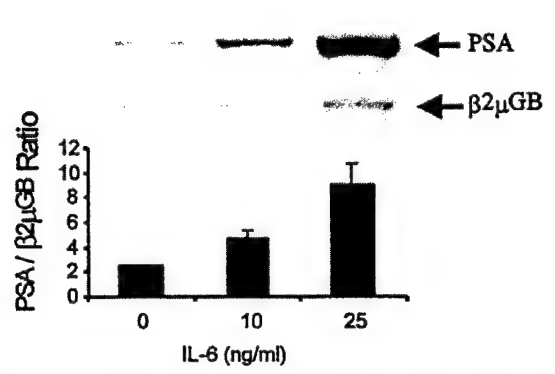

Fig. 1 IL-6 induces PSA mRNA levels from LNCaP cells in the absence of androgen. LNCaP cells were plated to a 60% confluence in charcoal-stripped media in 100-mm plates. Cells were then treated with 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, total RNA was collected and evaluated for the PSA mRNA levels by semiquantitative RT-PCR. PCR products were subjected to electrophoresis and measured by densitometry. The mRNA levels are expressed as the ratio of PSA: β2-microglobulin. The gel shown here is the result of a typical experiment. Results are shown as mean (± SE) from three independent experiments.

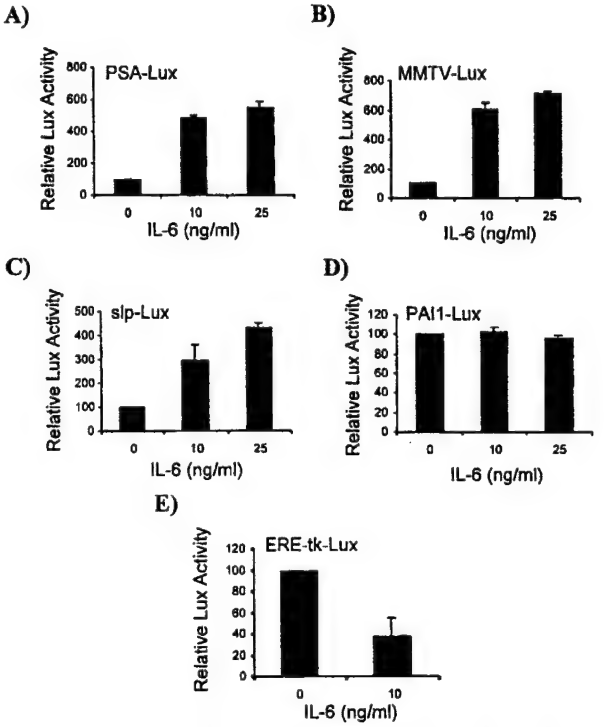

Fig. 2 IL-6 activates androgen-responsive promoters in the absence of androgen in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and one of the following: (*A*) p1.5kPSA-Lux, containing the proximal −1.5-k bp of the PSA promoter); (*B*) pMMTV-Lux, containing 150 bp of the MMTV promoter; (*C*) pc′Δ9(slp)-Lux, containing −900 bp of the SLP promoter; (*D*) pPAI-1-Lux, containing a human type-1 plasminogen-activator-inhibitor gene promoter (−800/+22); or (*E*) pERE-tk-Lux, containing tandem repeat of estrogen response elements upstream of the thymidine kinase promoter. Cells were then incubated in charcoal-stripped medium with the addition of 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

gy), diluted 1:500 in 1% BSA, 5% NFDM, and 0.1% Tween 20 in PBS. The images were captured using a fluorescent microscope. A GFP-AR expression plasmid (a gift from Dr. M. Lu, Harvard University, Boston, MA) was transfected into PC3 cells. The fusion fluorescent protein was imaged by confocal microscopy.

RESULTS

IL-6 modulates both cell growth (5, 21, 25, 26) and the androgen regulatory signal pathway in LNCaP cells (18, 19). However, results regarding IL-6 expression and activity in LNCaP cells are rather inconsistent (27). To determine whether IL-6 induces an androgen-like response in the LNCaP model system in our hands, the ability of increasing doses of IL-6 to modulate steady-state mRNA expression of the androgen-responsive PSA (28) in LNCaP cells was determined. In the absence of androgens, IL-6 caused a dose-dependent increase of PSA mRNA levels (Fig. 1). The mean PSA mRNA levels increased ~2- and 3.5-fold at 10 and 25 ng/ml of IL-6, respectively. Thus, IL-6 increases steady-state mRNA expression of an androgen-responsive gene, which is consistent with the possibility that it may activate the AR.

To determine whether IL-6 increased steady state mRNA levels of androgen-responsive genes through increasing their transcription, the ability of IL-6 to activate that PSA promoter was determined. IL-6 induced more than a 4-fold increase of PSA promoter activity (Fig. 2*A*). To next evaluate whether IL-6-mediated transactivation extended to other androgen-responsive promoters, we measured the ability of IL-6 to activate the MMTV(22) and the mouse SLP (24) promoters. IL-6 (10–25 ng/ml) transactivated both the MMTV and SLP promoters approximately 6-fold and 3- to 4-fold, respectively (Fig. 2*B* and 2*C*) demonstrating that IL-6 can induce a variety of androgen-responsive genes. To determine whether IL-6-mediated transactivation was a nonspecific phenomenon, we evaluated the ability of IL-6 to induce several androgen nonresponsive genes; the human type-1 plasminogen activator inhibitor gene promoter and the thymidine kinase (tk) promoter downstream of a tandem ER response elements. In contrast to the androgen-responsive genes, these promoters were not activated by IL-6 (Fig. 2*D* and 2*E*). Taken together, these data suggest that IL-6 transactivates androgen-responsive genes with some degree of specificity.

The observation that IL-6 activated several different androgen-responsive genes suggests that there is a common mechanism through which IL-6 mediates this effect. A candidate target for IL-6-mediated transactivation of androgen-responsive promoters is the AR. Thus, to test if IL-6 transactivates androgen-responsive promoters through the AR, we incubated the cells with BIC (Casodex), an antiandrogen that is able to completely abolish AR function (29). BIC completely blocked the IL-6-mediated induction of the PSA and MMTV promoters (Fig. 3*A*) suggesting that IL-6 induces these genes through the AR. To further support IL-6's requirement for AR, the ability of

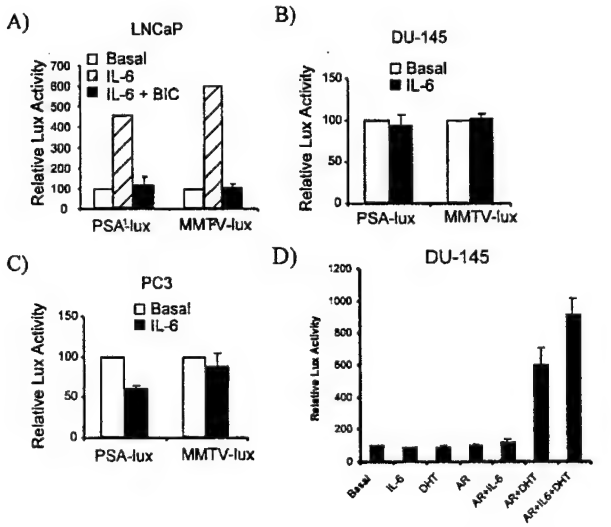

Fig. 3 IL-6-induced transcriptional activity requires an active AR. (*A*) LNCaP cells, (*B*) DU-145 cells, and (*C*) PC3 cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and either p1.5kPSA-Lux or pMMTV-Lux. The cells were then untreated (*Basal*) or treated with either IL-6 (10 ng/ml) or IL-6 (10ng/ml) and 20 μM BIC for 24 h as indicated. After incubation, lux levels were measured using a luminometer. *D*, DU-145 cells were cotransfected with pRL-CMV and p1.5kPSA-Lux. *Lanes 4–7*, cells were additionally cotransfected with 50 ng of pCMV-AR (*AR*). Cells were then incubated in charcoal-stripped media with the addition of 10 ng/ml IL-6, or 10 nM DHT, or both (as indicated) for 24 h. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

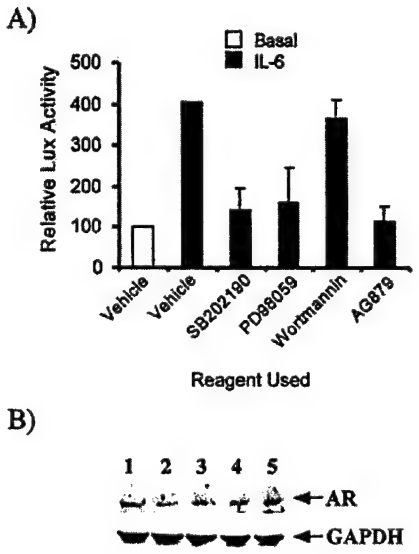

Fig. 4 PSA promoter induction by IL-6 was blocked by specific inhibitors to signal transducers. *A*, LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and p1.5kPSA-Lux. The cells were then incubated with vehicle, SB202190 (5 μM), PD98059 (15 μM), wortmannin (100 nM), or tyrphostin AG879 (10 μM) for 30 min prior to IL-6 administration (10 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments. *B*, the immunoblot of AR and GAPDH from 40 μg of cell lysate. LNCaP cells were cultured in medium containing IL-6 (*Lane 1*) or IL-6 plus pretreatment of the inhibitor reagents described above (*Lanes 2–5*, respectively).

IL-6 to activate the PSA and MMTV promoters in two AR-negative CaP cell lines that express the IL-6 receptor α and β (gp 130) chains (4, 5, 21), DU-145 and PC3, was determined. IL-6 did not induce PSA or MMTV promoter activity in these cell lines (Fig. 3*B* and 3*C*). When AR was transiently expressed in DU-145 cells, IL-6 alone still did not induce the PSA promoter (Fig. 3*D*, *Lane 5*) although DHT alone did (Fig. 3*D*, *Lane 6*). However, IL-6 increased the androgen-induced PSA promoter activity (Fig. 3*D*, *Lane 7*). Similar results were obtained for activation of the MMTV promoter (data not shown). Collectively, these data suggest that AR is required, but not sufficient, for the IL-6-mediated activation of androgen-responsive promoters.

Several nonsteroidal substances induce AR activity through a variety of signal transduction pathways (18, 30–34). IL-6 activates PI-3 kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Furthermore, IL-6 requires the growth factor receptors ErbB2 and -3 for signaling in LNCaP cells (20). To determine whether IL-6-induced androgen-responsive gene activation is mediated through these signal transduction pathways, the ability of kinase inhibitors to abrogate IL-6-mediated activation of the PSA promoter was evaluated. SB202190 (a p38 MAPK inhibitor), PD98059 (a MEK1/2 inhibitor), and tyrphostin AG879 (a ErbB2 MAPK inhibitor) but not wortmannin (a PI-3-kinase inhibitor), blocked IL-6-mediated induction of the PSA promoter activity (Fig. 4*A*). LY294002 (a PI-3-kinase inhibitor) has been shown to cause program cell death to LNCaP (37). To ensure that the negative result that we obtained in Fig. 4*A* was not derived from cell death, we examined the cell viability by trypan blue exclusion on cells that followed the same treatment. We found no significant variation of the living cell number among different treatment group (data not shown). Because, in the report of Carsen *et al.*, addition of growth factor or serum in addition to LY294002 protected the LNCaP cells from apoptosis, it is plausible that the addition of IL-6 after cells were given the inhibitors may save cells from the cytotoxic effect. Also, to ensure that this response was not attributable to kinase inhibitor-mediated alteration of AR levels, we measured AR protein in the cells. The level of AR from cells treated with IL-6 and kinase inhibitors was the same as to that of IL-6 alone (Fig. 4*B*), except for a slight reduction of AR level from cells treated with SB202190 (Fig. 4*B*, *Lane 2*). Because MAPK inhibition blocked IL-6-mediated activation of the PSA promoter, we next assessed whether activation of MAPK could induce PSA promoter expression. Accordingly, we treated cells with EGF (a MAPK activator known to activate ER-dependent promoter activities; Ref. 38). EGF (100 ng/ml) did not induce PSA promoter activity in either the absence or the presence of transgenic AR in DU145 cells (data not shown). However, EGF (100 ng/ml) induced ERE-tk-Lux activity by 30-fold in the presence of transgenic ERα, which demonstrated that EGF was functional (data not shown). Together, these data suggest that MAPK activation is

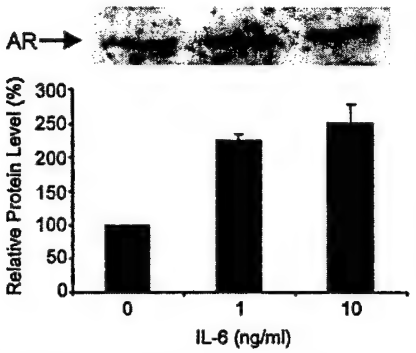

Fig. 5 Total AR levels from LNCaP cells are up-regulated by IL-6. LNCaP cells were treated with 0, 1, or 10 ng/ml of IL-6 for 24 h in charcoal-stripped media. Total cellular protein was then collected and subjected to Western blot analysis (200 μg/lane) using an antihuman AR NH_2-terminal antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as mean (±SE) percentage of AR protein level relative to untreated cells, which was set as 100%, from three independent experiments.

necessary, but not sufficient, for IL-6-mediated activation of the PSA promoter.

Hobisch *et. al.* reported that IL-6 induces LNCaP cell androgen responsiveness in the presence of low doses of androgen (18). One mechanism, through which IL-6 may increase androgen responsiveness in an AR-dependent fashion, as the current data demonstrate, is through induction of AR expression. Thus, the ability of IL-6 to increase AR expression in LNCaP cells was determined. The average AR protein amount doubled in the presence of 1 ng/ml of IL-6, whereas a 2.5-fold increase of mean AR level was observed when the IL-6 concentration was increased an order of magnitude (Fig. 5, *Lanes 2* and *3*). These data are consistent with the possibility that IL-6 sensitizes LNCaP cells to androgen through increased AR expression. However, the AR typically translocates to the nucleus to exert its function on gene expression (39). Accordingly, we determined whether IL-6 induces nuclear AR translocation. In the absence of DHT or IL-6, AR was detected primarily in the cytoplasm (Fig. 6, *A* and *D*). IL-6 induced a nuclear AR pattern similar to that of DHT-induced nuclear AR expression (compare Fig. 6, *B*, *C*, *E*, and *F*). In agreement with the immunofluorescent staining and the GFP tagging results, IL-6 increased AR levels in nuclear extract from LNCaP cells (Fig. 6*G*). Taken together, these data suggest that IL-6 induces androgen-responsiveness through increasing total AR protein levels that results in increased nuclear AR.

It is plausible that IL-6 induces AR expression through transcriptional or translational mechanisms. To determine whether IL-6 increases AR expression levels through transcriptional mechanisms, the ability of IL-6 to modulate AR mRNA levels was initially determined. IL-6 at 10 and 25 ng/ml increased steady-state AR mRNA levels 3-fold and 4-fold, respectively (Fig. 7). To evaluate whether IL-6 increases steady-state AR mRNA levels through transcription, the ability of IL-6 to activate the AR promoter was determined. IL-6 activated the AR promoter in a dose-dependent fashion (Fig. 8). Specifically, IL-6 at 5 and 25 ng/ml induced 3- and 3.5-fold activation, respectively, of the AR promoter (Fig. 8, *Lanes 2* and *3* compared with *Lane 1*). These data correlated well with the magnitude of increased steady-state AR mRNA levels induced by IL-6. To determine whether the IL-6-induced increase of AR levels is sufficient to increase androgen activity in the absence of ligand, we overexpressed AR in LNCaP cells (Fig. 9, *Lanes 2* and *3*). Surprisingly, overexpression of AR diminished basal PSA mRNA levels (Fig. 9). These results demonstrate that increased AR levels alone are not sufficient to mediate IL-6 induction of androgen response in the absence of androgen, which suggests that IL-6 activates the AR in addition to increasing AR expression.

DISCUSSION

Induction of AR gene transcription is a relatively understudied area. Prior to the current study, only androgens were reported to activate the AR gene promoter. Thus, the present report is the first description of a non-androgen inducer of AR gene transcription, namely IL-6. In addition to increasing AR gene expression, this study demonstrated that IL-6 activates the AR in the absence of androgen. The ability of IL-6 to activate AR requires MAPK activity; however, MAPK alone is not sufficient to activate the AR. Taken together, these results suggest that IL-6 may promote androgen-independent prostate cancer progression through both increasing AR levels and increasing AR activity.

Elevated serum IL-6 expression is associated with the morbidity and progression of prostate cancer (40). Furthermore, IL-6 induces prostate cancer cell proliferation and protects prostate cancer cell lines from chemotherapeutics *in vitro* (41, 42). Our finding that IL-6 induced three different androgen-responsive promoters (PSA promoter, MMTV promoter, and SLP promoter) is consistent with a previous report that IL-6 activated an ARE-driven minimal promoter reporter vector (18). Our study extended this previous report by evaluating the affect of IL-6 on the ARE in the context of several natural androgen-responsive promoters, thus approximating the natural promoter activity better than isolated AREs. Our observations suggest that one mechanism through which IL-6 may contribute to prostate cancer progression is the ability to activate a general androgen response in prostate tumors. Such a response could be associated with increased tumor proliferation in the absence of androgens, which would contribute to the development of androgen independence.

The observation that IL-6 activated several androgen-responsive promoters but not androgen-nonresponsive promoters suggests that IL-6 has a degree of specificity for inducing androgen-like response. One potential mechanism that could account for such a response is the induction of AR activity. The observations that BIC blocked the effect of IL-6 and that IL-6 could not activate the PSA and MMTV promoters in AR-negative CaP cells support this hypothesis. Two non-mutually exclusive mechanisms that could account for the ability of IL-6 to increase AR action are increased AR levels and increased AR function.

The observation that IL-6 induced androgen-responsive genes in the absence of androgens through an AR-dependent mechanism suggested that IL-6 activates AR function. This

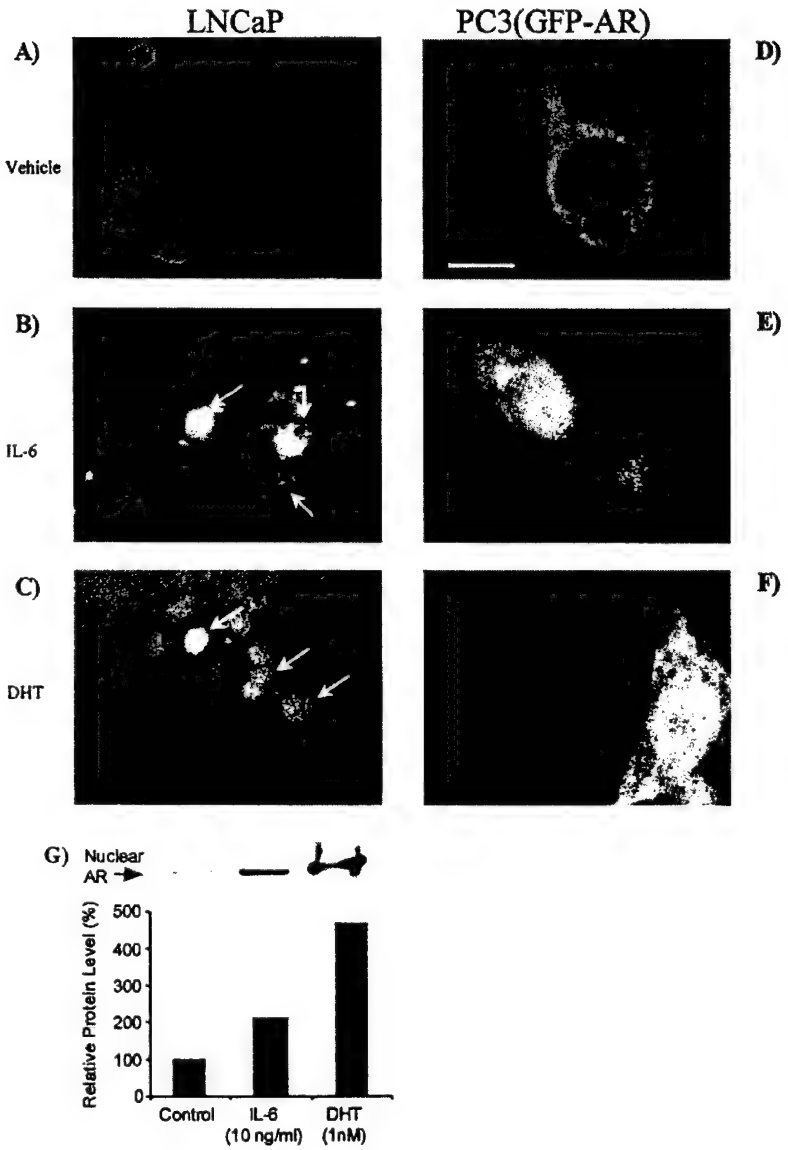

Fig. 6 IL-6 increases nuclear AR levels in prostate cancer cells. LNCaP cells and GFP-AR-expressing PC3 cells were incubated with: *A* and *D*, vehicle alone; *B* and *E*, IL-6 (10 ng/ml); or *C* and *F*, DHT (1 nM). After 24 h, they were subjected to immunofluorescent staining with an anti-AR antibody or confocal microscopy. *Arrows*, nuclear staining. *A*, ×400; *B*, ×630; *C*, ×400. *Bar*, 15 μm. *G*, LNCaP cells were treated with medium alone, IL-6 (10 ng/ml), or DHT (1 nM) for 24 h; then nuclear proteins were collected and subjected to Western blot analysis (20 μg/lane) and probed with anti-AR antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as percentage of AR protein relative to untreated cells, which was set at 100%.

observation is consistent with several other nonandrogenic compounds that have been reported to stimulate AR. For example, several substances that bind to membrane receptors up-regulate the activity of AR (30–34). Furthermore, that IL-6 increased nuclear AR in the absence of androgen suggests that IL-6 may mediate its effect in part through unmasking the nuclear localization sequence. However, our observation that IL-6 did not induce androgen-responsive genes in the DU145 cells transfected with the AR is in apparent conflict with these findings. Taken together, these observations suggest that the AR is required but not sufficient for IL-6 to mediate androgen-like activity. It is possible that DU145 cells are deficient in some cofactor required for IL-6 to mediate its androgen-like activity.

Several signal transduction molecules are potential mediators of the ability of IL-6 to activate AR. For example, the observations that several MAPK inhibitors blocked IL-6-mediated androgen-responsive promoter activation suggest that IL-6 induces AR activity through a MAPK-dependent pathway. These data are consistent with the ability of IL-6 to induce the Ras signal transduction pathways (43), which depends on Raf, MEK, and MAPK. These data are also consistent with the previous report that IL-6 requires the growth factor receptors ErbB2 and -3 to mediate signaling in LNCaP cells (20). However, the observation that the PI-3-kinase inhibitor, wortmannin, had no effect on IL-6-mediated PSA promoter induction suggests that this pathway is not important for androgen responsiveness, although IL-6 activates the PI-3-kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Finally, the recent report that the inhibition of IL-6-mediated activation of STAT3 diminishes the ability of IL-6 to induce androgen-like activity suggests that the Jak/STAT pathway is an important component of IL-6-mediated activation of the AR

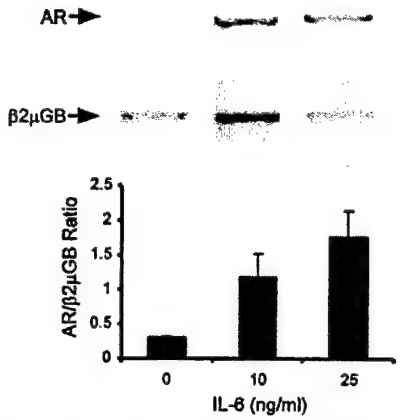

Fig. 7 IL-6 induces AR mRNA levels in LNCaP cells. LNCaP cells were treated with IL-6 (0, 10, and 25 ng/ml) for 24 h. Total RNA was collected and subjected to semiquantitative RT-PCR using primers for AR (*AR*) and β2-microglobulin (*β2μGB*). PCR products were measured by densitometry. Data are reported as the mean (± SE) ratio of AR: β2μGB mRNA from three independent experiments. The gel shown is the result of a typical experiment.

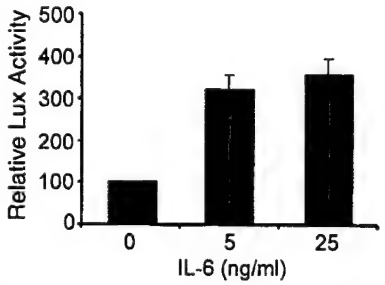

Fig. 8 IL-6 stimulates AR gene promoter activity in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and the reporter plasmid, p2.3kAR-Lux. Cells were then treated with IL-6 (0, 5, 25 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (±SE) lux activity relative to the untreated cells, which was set as 100%, from three independent experiments.

(19). These previous reports, taken together with our current data, suggest that both MAPK and Jak/STAT signal transduction pathways are required to mediate the effect of IL-6 on AR function.

In addition to increased AR function, the finding that IL-6 increased AR expression at both mRNA and protein levels suggests that IL-6 enhances androgen activity by up-regulating the AR levels. This is consistent with the heterogeneous expression of AR that occurs as prostate cancer progresses (44–46). Furthermore, it has pathophysiological implications because there are low levels of androgens even after androgen ablation therapy (47). Specifically, if IL-6 increases AR levels in prostate cancer cells, then it follows that these cells may have increased sensitivity to the low levels of androgens in the androgen-ablated patient, enabling these cells to respond to androgens. In effect, what appears to be an androgen-independent tumor, based on its progression in an androgen-ablated patient, would

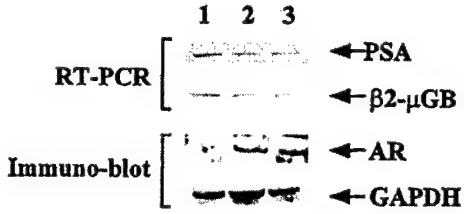

Fig. 9 Increased AR levels in LNCaP cells cause reducing PSA expression. LNCaP cells were transfected with empty vector (*Lane 1*), 0.4 μg or 2 μg of AR plasmid (*Lanes 2* and *3*). Total RNA was collected and subjected to semiquantitative RT-PCR using primers for PSA and β2-microglobulin (β2μGB). PCR products were resolved by electrophoresis. Protein from the same cell lysate 40 μg were resolved by SDS-PAGE gel electrophoresis followed by AR and GAPDH immunoblotting.

really be hypersensitive to androgens. This hypothesis is consistent with the fact that the LNCaP cells, which are androgen responsive, were originally isolated from a patient in whom androgen ablation failed (16, 17).

The clinical observations that elevated IL-6 levels are frequently associated with androgen-independent prostate cancer have predicted an important role for IL-6 signaling in prostate cancer androgen-independent progression (40, 48–51). For example, serum IL-6 levels were shown to predict survival in prostate cancer patients (49). IL-6 levels in the serum of these patients range between 10 and 700 pg/ml, with occasional findings of >1 ng/ml (40, 48–51). In our study, we used nanogram levels of IL-6, which may reflect the IL-6 levels found in the tumor microenvironment. This postulation is supported by the observations that several prostate cancer cell lines produce nanogram levels of IL-6[4] (5, 52). However, because of the wide range of IL-6 levels in men afflicted with prostate cancer, the *in vitro* application of IL-6 at the nanogram level requires cautious interpretation in terms of its bearing on *in vivo* pathophysiology. Moreover, from our data, which showed that IL-6 very likely contributes to the growth of advanced-prostate-cancer growth and that BIC abolishes the effect of IL-6, one might expect that this antiandrogen will be a powerful therapeutic agent in treating the advanced-prostate-cancer patient. Unfortunately, trials of BIC monotherapy have not demonstrated efficacy in the clinical setting (reviewed in Ref. 53). Because of the fact that the cancer tissue is heterogeneous, it is likely that there is a subpopulation of cancer cells that can survive via alternative mechanisms to which IL-6 may contribute independent of the AR signaling pathway.

The demonstration of IL-6-induced activation of the AR promoter and the subsequent increase of AR mRNA and protein is the first report of non-androgen-mediated induction of the human AR promoter. Other than IL-6, only androgens have been reported to activate the human AR promoter (54–56). Grad *et al.* (57) demonstrated the regulatory motif within the AR gene-coding region required for AR auto-up-regulation. Whether or not IL-6 uses this same motif is currently unknown.

[4] Unpublished observations.

Furthermore, the *trans*-acting factors and *cis*-acting promoter sites through which IL-6 mediates induction of the AR promoter are currently unknown. The induction of AR mRNA and protein expression described thus far has been rare (58–60). Thus, our data provide a new mechanism through which AR levels are controlled.

Advanced CaP is associated with increased serum IL-6 levels (50). The source of IL-6 in the CaP patients is not known. However, we have previously demonstrated that androgen down-regulates IL-6 expression in LNCaP cells (61). Furthermore, we and others have shown that orchiectomy induces IL-6 expression in mice (62, 63). Thus, it follows that androgen deprivation may account for the increased serum IL-6 levels observed in patients with advance prostate cancer. These observations, taken together with results from the present study, suggest that androgen deprivation may induce IL-6 expression in prostate cells, which, in turn, will induce androgen-like activity in the CaP cells. This could lead to androgen-independent tumor growth. Intriguingly, it is possible that, as IL-6 induces androgen-like activity, this will in turn create a negative feedback on IL-6 production.

In summary, our study provides evidence that IL-6 increases androgen-like action by up-regulating the AR expression in CaP cells. Furthermore, our data suggest that IL-6 activates the AR in the absence of androgen. Together, these mechanisms can account for the contribution of IL-6 to the progression of prostate cancer. Furthermore, these results suggest that androgen-deprivation therapy may promote the progression of CaP to an androgen-independent state through increasing expression of IL-6.

Acknowledgments

We thank Drs. Atsushi Mizokami, Diane Robins, Ronald Koenig, Thomas Gelehrter, Michael Lu, and Pei-Wen Hsiao for supplying us with the reporter reagents. We also thank Dr. Jill Macoska for her help with the fluorescent microscopy and Dorothy Sorenson for her help with the confocal microscopy.

REFERENCES

1. Kyprianou, N., English, H. F., and Isaacs, J. T. Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res., *50:* 3748–3753, 1990.

2. Isaacs, J. T., Schulze, H., and Coffey, D. S. Development of androgen resistance in prostatic cancer, Prog. Clin. Biol. Res., 21–31, 1987.

3. Knabbe, C., Kellner, U., Schmahl, M., and Voigt, K. D. Growth factors in human prostate cancer cells: implications for an improved treatment of prostate cancer. J. Steroid Biochem. Mol. Biol., *40:* 185–192, 1991.

4. Siegall, C. B., Nordan, R. P., FitzGerald, D. J., and Pastan, I. Cell-specific toxicity of a chimeric protein composed of interleukin-6 and *Pseudomonas* exotoxin (IL6-PE40) on tumor cells. Mol. Cell. Biol., *10:* 2443–2447, 1990.

5. Okamoto, M., Lee, C., and Oyasu, R. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells *in vitro*. Cancer Res., *57:* 141–146, 1997.

6. Ershler, W. B., and Keller, E. T. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu. Rev. Med., *51:* 245–270, 2000.

7. Lord, K. A., Abdollahi, A., Thomas, S. M., DeMarco, M., Brugge, J. S., Hoffman-Liebermann, B., and Liebermann, D. A. Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. Mol. Cell. Biol., *11:* 4371–4379, 1991.

8. Nakajima, K., and Wall, R. Interleukin-6 signals activating *junB* and *TIS11* gene transcription in a B-cell hybridoma. Mol. *Cell.* Biol., *11:* 1409–1418, 1991.

9. Yuan, J., Wegenka, U. M., Lutticken, C., Buschmann, J., Decker, T., Schindler, C., Heinrich, P. C., and Horn, F. The signalling pathways of interleukin-6 and γ interferon converge by the activation of different transcription factors which bind to common responsive DNA elements. Mol. Cell. Biol., *14:* 1657–1668, 1994.

10. Siegsmund, M. J., Yamazaki, H., and Pastan, I. Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia. J. Urol., *151:* 1396–1399, 1994.

11. Hobisch, A., Rogatsch, H., Hittmair, A., Fuchs, D., Bartsch, G., Jr., Klocker, H., Bartsch, G., and Culig, Z. Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. J. Pathol., *191:* 239–244, 2000.

12. Evans, R. M. The steroid and thyroid hormone receptor superfamily. Science (Wash. DC)., *240:* 889–895, 1988.

13. Trapman, J., and Brinkmann, A. O. The androgen receptor in prostate cancer. Pathol. Res. Pract., *192:* 752–760, 1996.

14. Shain, S. A., Gorelic, L. S., Klipper, R. W., Ramzy, I., Novicki, D. E., Radwin, H. M., and Lamm, D. L. Inability of cytoplasmic or nuclear androgen receptor content or distribution to distinguish benign from carcinomatous human prostate. Cancer Res., *43:* 3691–3695, 1983.

15. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.

16. Schuurmans, A. L., Bolt, J., Veldscholte, J., and Mulder, E. Stimulatory effects of antiandrogens on LNCaP human prostate tumor cell growth. EGF-receptor level and acid phosphatase secretion, J. Steroid Biochem. Mol. Biol., *37:* 849–853, 1990.

17. Lee, C., Sutkowski, D. M., Sensibar, J. A., Zelner, D., Kim, I., Amsel, I., Shaw, N., Prins, G. S., and Kozlowski, J. M. Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells. LNCaP, by dihydrotestosterone, Endocrinology, *136:* 796–803, 1995.

18. Hobisch, A., Eder, I. E., Putz, T., Horninger, W., Bartsch, G., Klocker, H., and Culig, Z. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res., *58:* 4640–4645, 1998.

19. Chen, T., Wang, L. H., and Farrar, W. L. Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. Cancer Res., *60:* 2132–2135, 2000.

20. Qiu, Y., Ravi, L., and Kung, H. J. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. Nature (Lond.), *393:* 83–85, 1998.

21. Mori, S., Murakami-Mori, K., and Bonavida, B. Interleukin-6 induces G_1 arrest through induction of p27(Kip1), a cyclin-dependent kinase inhibitor, and neuron-like morphology in LNCaP prostate tumor cells. Biochem. Biophys. Res. Commun., *257:* 609–614, 1999.

22. Cato, A. C., Henderson, D., and Ponta, H. The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J., *6:* 363–368, 1987.

23. Hsiao, P. W., Lin, D. L., Nakao, R., and Chang, C. The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J. Biol. Chem., *274:* 20229–20234, 1999.

24. Adler, A. J., Scheller, A., and Robins, D. M. The stringency and magnitude of androgen-specific gene activation are combinatorial functions of receptor and nonreceptor binding site sequences. Mol. Cell. Biol., *13:* 6326–6335, 1993.

25. Qiu, Y., Robinson, D., Pretlow, T. G., and Kung, H. J. Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of

phosphatidylinositol 3′-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc. Natl. Acad. Sci. USA, *95:* 3644–3649, 1998.

26. Chung, T. D., Yu, J. J., Kong, T. A., Spiotto, M. T., and Lin, J. M. Interleukin-6 activates phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. Prostate, *42:* 1–7, 2000.

27. Smith, P. C., Hobisch, A., Lin, D-L., Culig, Z., and Keller, E. T. Interleukin-6 and prostate cancer progression, Cytokine Growth Factor Rev, *12:* 33–40, 2001.

28. Cleutjens, K. B., van Eekelen, C. C., van der Korput, H. A., Brinkmann, A. O., and Trapman, J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J. Biol. Chem., *271:* 6379–6388, 1996.

29. Bratoeff, E., Ramirez, E., Murillo, E., Flores, G., and Cabeza, M. Steroidal antiandrogens and 5α-reductase inhibitors. Curr. Med. Chem., *6:* 1107–1123, 1999.

30. Ikonen, T., Palvimo, J. J., Kallio, P. J., Reinikainen, P., and Janne, O. A. Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation. Endocrinology, *135:* 1359–1366, 1994.

31. Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res., *54:* 5474–5478, 1994.

32. Nazareth, L. V., and Weigel, N. L. Activation of the human androgen receptor through a protein kinase A signaling pathway. J. Biol. Chem., *271:* 19900–19907, 1996.

33. Darne, C., Veyssiere, G., and Jean, C. Phorbol ester causes ligand-independent activation of the androgen receptor. Eur. J. Biochem., *256:* 541–549, 1998.

34. Sadar, M. D. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J. Biol. Chem., *274:* 7777–7783, 1999.

35. Takahashi-Tezuka, M., Hibi, M., Fujitani, Y., Fukada, T., Yamaguchi, T., and Hirano, T. Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. Oncogene, 14: 2273–2282, 1997.

36. Lin, J., Adam, R. M., Santiestevan, E., and Freeman, M. R. The phosphatidylinositol 3′-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. Cancer Res., *59:* 2891–2897, 1999.

37. Carson, J. P., Kulik, G., and Weber, M. J. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. Cancer Res., 59: 1449–1453, 1999.

38. Smith, C. L. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol. Reprod., *58:* 627–632, 1998.

39. O'Malley, B. W., Tsai, S. Y., Bagchi, M., Weigel, N. L., Schrader, W. T., and Tsai, M. J. Molecular mechanism of action of a steroid hormone receptor. Recent Prog. Horm. Res., *47:* 1–24, 1991.

40. Twillie, D. A., Eisenberger, M. A., Carducci, M. A., Hseih, W. S., Kim, W. Y., and Simons, J. W. Interleukin-6: a candidate mediator of human prostate cancer morbidity. Urology, *45:* 542–549, 1995.

41. Lou, W., Ni, Z., Dyer, K., Tweardy, D. J., and Gao, A. C. Interleukin-6 induces prostate cancer cell growth accompanied by activation of stat3 signaling pathway. Prostate, *42:* 239–242, 2000.

42. Borsellino, N., Bonavida, B., Ciliberto, G., Toniatti, C., Travali, S., and D'Alessandro, N. Blocking signaling through the Gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumor cells to etoposide and cisplatin-mediated cytotoxicity. Cancer (Phila.), *85:* 134–144, 1999.

43. Satoh, T., Nakafuku, M., and Kaziro, Y. Function of Ras as a molecular switch in signal transduction. J. Biol. Chem., *267:* 24149–24152, 1992.

44. Prins, G. S., Sklarew, R. J., and Pertschuk, L. P. Image analysis of androgen receptor immunostaining in prostate cancer accurately predicts response to hormonal therapy. J. Urol., *159:* 641–649, 1998.

45. Koivisto, P., Kononen, J., Palmberg, C., Tammela, T., Hyytinen, E., Isola, J., Trapman, J., Cleutjens, K., Noordzij, A., Visakorpi, T., and Kallioniemi, O. P. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res., *57:* 314–319, 1997.

46. Hobisch, A., Culig, Z., Radmayr, C., Bartsch, G., Klocker, H., and Hittmair, A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res., *55:* 3068–3072, 1995.

47. Oefelein, M. G. Serum testosterone-based luteinizing hormone-releasing hormone agonist redosing schedule for chronic androgen ablation: a Phase I assessment. Urology., *54:* 694–699, 1999.

48. Wise, G. J., Marella, V. K., Talluri, G., and Shirazian, D. Cytokine variations in patients with hormone treated prostate cancer. J. Urol., *164:* 722–725, 2000.

49. Nakashima, J., Tachibana, M., Horiguchi, Y., Oya, M., Ohigashi, T., Asakura, H., and Murai, M. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. Clin. Cancer Res., *6:* 2702–2706, 2000.

50. Adler, H. L., McCurdy, M. A., Kattan, M. W., Timme, T. L., Scardino, P. T., and Thompson, T. C. Elevated levels of circulating interleukin-6 and transforming growth factor-β1 in patients with metastatic prostatic carcinoma. J. Urol., *161:* 182–187, 1999.

51. Akimoto, S., Okumura, A., and Fuse, H. Relationship between serum levels of interleukin-6, tumor necrosis factor-α and bone turnover markers in prostate cancer patients. Endocr. J., *45:* 183–189, 1998.

52. Chung, T. D., Yu, J. J., Spiotto, M. T., Bartkowski, M., and Simons, J. W. Characterization of the role of IL-6 in the progression of prostate cancer. Prostate, *38:* 199–207, 1999.

53. Goa, K. L., and Spencer, C. M. Bicalutamide in advanced prostate cancer. A review. Drugs Aging, *12:* 401–422, 1998.

54. Takane, K. K., and McPhaul, M. J. Functional analysis of the human androgen receptor promoter. Mol. Cell. Endocrinol., *119:* 83–93, 1996.

55. Mizokami, A., and Chang, C. Induction of translation by the 5′-untranslated region of human androgen receptor mRNA, J. Biol. Chem., 269: 25655–25659, 1994.

56. Faber, P. W., van Rooij, H. C., Schipper, H. J., Brinkmann, A. O., and Trapman, J. Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. The role of Sp1. J. Biol. Chem., *268:* 9296–9301, 1993.

57. Grad, J. M., Dai, J. L., Wu, S., and Burnstein, K. L. Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. Mol Endocrinol., *13:* 1896–1911, 1999.

58. Wiren, K., Keenan, E., Zhang, X., Ramsey, B., and Orwoll, E. Homologous androgen receptor up-regulation in osteoblastic cells may be associated with enhanced functional androgen responsiveness. Endocrinology, *140:* 3114–3124, 1999.

59. Kerr, J. E., Allore, R. J., Beck, S. G., and Handa, R. J. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus. Endocrinology, *136:* 3213–3221, 1995.

60. Gonzalez-Cadavid, N., Vernet, D., Fuentes Navarro, A., Rodriguez, J. A., Swerdloff, R. S., and Rajfer, J. Up-regulation of the levels of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis. Mol. Cell. Endocrinol., *90:* 219–229, 1993.

61. Keller, E. T., Chang, C., and Ershler, W. B. Inhibition of NFκB activity through maintenance of IκBα levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J. Biol. Chem., *271:* 26267–26275, 1996.

62. Zhang, J., Pugh, T. D., Stebler, B., Ershler, W. B., and Keller, E. T. Orchiectomy increases bone marrow interleukin-6 levels in mice. Calcif. Tissue Int., *62:* 219–226, 1998.

63. Bellido, T., Jilka, R. L., Boyce, B. F., Girasole, G., Broxmeyer, H., Dalrymple, S. A., Murray, R., and Manolagas, S. C. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. J. Clin. Investig., *95:* 2886–2895, 1995.

ELSEVIER

Cytokine and Growth Factor Reviews 12 (2001) 33–40

Cytokine Growth Factor Reviews

www.elsevier.com/locate/cytogfr

Mini Review

Interleukin-6 and prostate cancer progression

Peter C. Smith [a], Alfred Hobisch [b], Din-Lii Lin [a], Zoran Culig [b], Evan T. Keller [a,*]

[a] *Unit for Laboratory Animal Medicine, Institute of Gerontology and Connective Tissue Oncology Program, Room 5304 CCGCB, 1500 E. Medical Center Drive, University of Michigan, Ann Arbor, MI 48109-0940, USA*

[b] *Department of Urology, University of Innsbruck, A-6020 Innsbruck, Austria*

Abstract

Prostate cancer, while initially dependent on androgens for proliferation, progresses to an androgen-independent state. Evidence has been accumulating that interleukin-6 (IL-6) may contribute to prostate cancer progression. Serum levels of IL-6 correlate with prostate tumor burden and patient morbidity. The prostate tissue itself appears to be a source of IL-6 and its receptor. Furthermore, experimental data suggest that IL-6 is an autocrine and paracrine growth factor for androgen-independent prostate cancer cell lines. For example, inhibition of IL-6, with anti-IL-6 antibody, sensitizes androgen-independent prostate cancer cells to chemotherapeutic agents in vitro. Finally, IL-6 activates a variety of signal transduction cascades, some which stimulate androgen receptor activity, in prostate cancer cells. These data suggest that targeting IL-6 may have multiple benefits in prostate cancer patients.

Keywords: Cytokine; Androgen receptor; Signal transduction; Chemotherapy; Androgen independence

1. Introduction

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the US. In 1999, it was estimated that 179 300 patients were diagnosed with prostate cancer, and 37 000 patients died from the disease [1]. The increased incidence of prostate cancer in this country is unparalleled by any other tumor in the last 20 yr, and associated mortality has steadily increased [2].

Prostate cancer initially occurs as an androgen-dependent tumor. Thus, androgen-deprivation is a commonly used therapeutic strategy for prostate cancer. While the initial response rate is excellent, the cancer eventually recurs in the androgen-deplete state. The tumor, now termed androgen independent, typically progresses resulting in death of the patient. The mechanisms through which androgen independence develops are unknown. However, putative mechanisms can be divided into those that are dependent on activation of the androgen receptor (AR), either through ligand-dependent (i.e. androgen) or ligand-independent methods [3], or those mechanisms that activate prostate cancer proliferation through non-AR-dependent growth factors.

Among the many putative prostate cancer growth factors is the cytokine, interleukin-6 (IL-6). IL-6 has many physiologic roles and has been implicated in a number of pathophysiologic processes. A variety of tumor types are stimulated by IL-6, including melanoma, renal cell carcinoma, Kaposi's sarcoma, ovarian carcinoma, lymphoma and leukemia, multiple myeloma, and prostate carcinoma [4]. In the last few years, evidence has been accumulating that IL-6 may contribute to the progression of prostate cancer. The purpose of this article is to review the biology of IL-6 and its receptor, and to summarize information from the literature concerning their association with prostate cancer and their potential role in its pathophysiology.

2. Biology of IL-6 and its receptor

2.1. Interleukin-6

IL-6 is a 21–28 Kd protein containing 184 amino acids following cleavage of a 28 aa signal peptide [5].

* Corresponding author. Tel.: +1 734 615 0280; fax: +1 734 936 9220.

E-mail address: etkeller@umich.edu (E.T. Keller).

1359-6101/01/$ - see front matter
PII: S1359-6101(00)00021-6

IL-6 belongs to the "IL-6 type cytokine" family that also includes leukemia inhibitory factor, IL-11, ciliary neurotrophic factor, cardiotrophin-1 and oncostatin M [6]. In the normal homeostatic state, IL-6 levels are typically very low. However, in response to the appropriate stimulus (e.g. inflammation), a wide variety of cells produce IL-6. Many physiologic functions are attributed to IL-6 including promotion of antibody production from B lymphocytes, modulation of hepatic acute phase reactant synthesis, promotion of osteoclastic-mediated bone resorption, and induction of thrombopoiesis [7].

2.2. IL-6 receptor and signal transduction

IL-6 mediates its activity through the IL-6 receptor complex, which is composed of two components; an 80 Kd transmembrane receptor (IL-6Rp80, IL-6R, α-subunit) that specifically binds IL-6, but has no signaling capability and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6R binding [8]. Other members of the IL-6 family of cytokines have specific α-subunits, but share gp130 as a component critical for signal transduction [9]. The human IL-6R consists of 468 aa, including a signal peptide of 19 aa, an extracellular region of 339 aa, and a membrane-spanning region of 28 aa. Due to *N*-glycosylation, the molecular weight of IL-6R is 80 kDa instead of 50 kDa as predicted from its aa sequence [10]. Gp130 consists of 918 aa, including a leader sequence of 22 aa, an extracellular region of 597 aa, a membrane-spanning region of 22 aa, and a cytoplasmic region of 277 aa [1]. IL-6 binds to the IL-6R with low affinity [12,13] and induces disulfide-linked homodimerization of gp130 [13]. Gp130 has no intrinsic IL-6 binding capacity, but its complex formation with the IL-6R promotes high affinity binding of IL-6 as well as signal transduction [11]. The resulting compound is a hexameric complex consisting of two molecules each of IL-6, IL-6Rp80, and gp130 [12]. The homodimerization of gp130 activates associated tyrosine kinases (TYK) that subsequently cause gp130 phosphorylation, thus initiating signal transduction [13]. Mutation analysis of the cytoplasmic region of gp130 has led to identification of multiple regions implicated in signal transduction [14]. This reflects the pleiotropic nature of IL-6, in that separate sets of signals appear to be required to regulate such a diverse array of genes and cellular responses. Multiple sites are phosphorylated on tyrosine residues of gp130; however, gp130 does not appear to have any inherent tyrosine kinase activity. Instead, gp130 associates with tyrosine kinases such as Janus kinase family members JAK/TYK [15] and possibly others such as the src-related tyrosine kinase HcK [16]. Tyrosine residues within receptors mediate signal transduction through the recruitment of either src homology 2 (SH2) domains or phosphotyrosine binding domains. Gp130 has six tyrosine residues in its cytoplasmic domain [17]. By using chimeric receptors, consisting of the extracellular domain of growth hormone receptor and the transmembrane and cytoplasmic domain of gp130 with progressive C-terminal truncations, Yamanaka et al. demonstrated that the tyrosine residue with the YXXQ motif of the membrane-proximal of gp130 could generate the signals for growth arrest, macrophage differentiation, down-regulation of c-myc and c-myb, induction of junB and IRF1 and STAT3 activation [18]. This observation was further confirmed by Tomida et al. who constructed chimeric receptors by linking the transmembrane and intracellular regions of mouse gp130 to the extracellular domains of the human granulocyte macrophage colony-stimulating factor receptor alpha and beta chains. Using the full-length cytoplasmic domain and mutants with progressive C-terminal truncations or point mutations, they showed that the two membrane-distal tyrosines with the YXXQ motif of gp130 were critical not only for STAT3 activation, but also for growth arrest and differentiation of cells [19]. Thus, certain tyrosine residues play pivotal roles in gp130 mediated signal transduction regulating cell growth, differentiation, and survival.

In addition to the transmembrane IL-6R, a soluble form of IL-6R (sIL-6R) exists. There is evidence that the soluble form is produced by either proteolytic cleavage of the 80 kDa subunit [20,21] or differential splicing of mRNA [22]. Several recent reports that describe a truncated form of IL-6 receptor mRNA identified through RT-PCR from a human hepatoma cell line (HepG3), mononuclear cells from inflammatory bowel disease patients, acute myeloblastic leukemia (AML) patient cell lines, and primary AML blast cells [23–25] support alternative splicing as a mechanism of sIL-6R production. Although the sIL-6R does not posses a transmembrane component, it can still bind to IL-6 and the ligand bound sIL-6R. IL-6 complex activates signal transduction and biological responses through membrane-bound gp130 [26]. Thus, IL-6 can still affect cells that do not express transmembrane IL-6R, as long as they express gp130.

A few signaling pathways for IL-6 have been described that may act simultaneously in certain types of cells or preferentially in others. Some of the IL-6 response genes are targets of the tyrosine phosphorylation pathway involving Janus kinases (JAK) and the signal transducer and activator of transcription (STAT) family of nuclear factors. There appears to be constitutive association between gp130 and JAK1, JAK2, and TYK2. Tyrosine kinase activity is activated secondary to gp130 dimerization induced by the binding of IL-6 to IL-6R, probably through conformational change of the receptor complex [27]. Following the phosphorylation of gp130, STAT family members associate with gp130

dimers and act as substrates for JAK/TYK. In the case of STAT3, tyrosine phosphorylation occurs at a single residue (Tyr705) that is located in a conserved SH2 domain [28]. Upon tyrosine phosphorylation, STATs (including STAT1, STAT3, and STAT5) translocate to the nucleolus as homodimers or heterodimers and bind to specific consensus DNA sequences of target-gene promoters and activate transcription [14,29–35]. In addition to tyrosine phosphorylation, STAT3 is phosphorylated at a single serine residue (Ser727) in response to IL-6 [36]. A recent report by Schuringa et al. suggests that, independent of ERK-1 or JNK-1, IL-6-induced STAT3 transactivation and phosphorylation involves a gp130-signaling cascade that includes Vav, Rac-1, MEKK and SEK-1/MKK-4 as signal transduction components [37].

Another signaling pathway of IL-6 involves the GTP-binding protein Ras, which may also be involved in other cytokine systems. GTP-binding motifs are present in the gp130 intracytoplasmic region; however, their precise role is unclear [11]. This Ras-dependent pathway includes intermediate steps involving Raf, MEK (MAP kinase kinase), and MAPK [38–41]. Following translocation into the nucleus, it is believed that MAP kinase activates the nuclear factor for IL-6 (NF-IL6) transcription factor to act on its target genes [42]. The binding activity of NF-IL6 is most likely induced by IL-6 through the increased expression of the NF-IL6 gene, rather than through post-translational modification [43]. Other serine/threonine protein kinases can also be activated by IL-6 [44]. Moreover, IL-6 also activates phosphatidylinositol (PI3)-kinase through the activation of the p-85 subunit of PI3-kinase and contributes to the complexity of the cellular response to this cytokine [45–47].

3. IL-6 and prostate cancer

3.1. IL-6 and clinical prostate cancer

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer [48–50]. Adler et al. [48] demonstrated that serum levels of IL-6 and transforming growth factor-β1 are elevated in patients with metastatic prostate cancer, and that these levels correlate with tumor burden as assessed by serum PSA or clinically evident metastases. In a similar fashion, Drachenberg et al. [51] reported elevated serum IL-6 levels in men with hormone-refractory prostate cancer compared to normal controls, benign prostatic hyperplasia, prostatitis, and localized or recurrent disease. These observations suggest that IL-6 may be a surrogate marker of the androgen independent phenotype.

IL-6 has been shown to be a candidate mediator of prostate cancer morbidity, and a candidate marker of disease activity for prospective clinical testing [50]. Signs of morbidity associated with human prostate cancer include anorexia, anemia, cachexia, asthenia, elevated acute phase proteins, hypoalbuminemia, edema, anergy, and diffuse bone pain [50], and unlike most solid tumors, prostate cancer can cause death without causing massive destruction to vital organs by space occupying metastatic lesions [52]. Twillie et al. hypothesized that death in some patients with advanced hormone refractory prostate cancer may be caused by, or hastened by circulatory exposure of ejaculate proteins which have a normal role in the genitourinary tract, but are pathologic when they enter the systemic circulation in large amounts [50]. IL-6 has been shown to be a mediator of experimental cachexia [53], is a well documented mediator of inflammation, and elevated levels of this cytokine have been associated with morbidity in a number of chronic diseases [53]. In their 1995 study, Twillie et al. demonstrated that IL-6 is a normal component of seminal plasma, and that elevated serum levels of IL-6 in prostate cancer patients were associated with certain clinical parameters of morbidity such as leukocytosis, anemia, hypercholesterolemia, and elevated serum lactate dehydrogenase levels. In addition to its role as a mediator of morbidity, IL-6 may also act as a growth factor, and protect prostate cancer cells from cell death induced by certain chemotherapeutic agents. IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and is associated with poor prognosis in several solid and hematopoietic neoplasms such as renal cell carcinoma, ovarian cancer, lymphoma, and melanoma [54]. Thus, taken together, these data provide a large body of evidence that IL-6 is associated with prostate cancer in the clinical arena.

3.2. Identification of IL-6 and IL-6R in human tissue samples

IL-6 is produced by a large number of cells. However, its secretion is generally tightly controlled, and serum levels in healthy individuals are very low [51]. In terms of prostatic disease, it has been demonstrated that normal prostate epithelial cells, cells derived from benign prostatic hyperplasia, and primary prostate tumors secrete IL-6 in cell culture [50]. Thus, the prostate cancer cells themselves may be the source of elevated IL-6 levels found in prostate cancer patients. In addition to IL-6, the IL-6R has been identified in human prostate carcinoma and benign prostatic hyperplasia [55]. Using slot-blot analysis with a probe that recognizes mRNA encoding the α-subunit, IL-6R expression was found in 78% of BPH and 100% of prostate carcinoma samples analyzed. Hobisch and Culig

demonstrated the expression of IL-6 and its receptor (Fig. 1) in malignant and normal prostate using immunohistochemistry [56]. In normal prostatic tissue, IL-6 immunoreactivity was predominantly confined to basal cells. In contrast, only a small percentage of glandular cells showed a positive immune reaction for this cytokine. Interestingly, there was no IL-6 reaction in stromal cells. However, IL-6 secretion was detected in the supernatants of prostatic fibroblasts and smooth muscle [56]. Based on these results, it was proposed that there is a high secretion rate of IL-6 in prostatic stromal cells and therefore it is not detectable in these cells by immunohistochemistry. An alternative explanation is that process of cell culture itself stimulated IL-6 expression in the primary cells cultures, thus resulting in secretion of IL-6 in the supernatant. IL-6 staining pattern changed in high-grade prostate intraepithelial neoplasia (PIN) lesions and in cancer tissue. In 11 of 23 Gleason patterns investigated, there were more than 50% of IL-6-positive tumor cells. This study revealed that the IL-6 receptor is expressed in normal prostate, high-grade PIN and cancer tissue [56]. In nonmalignant tissue, IL-6R was detected in both basal and secretory cells. All prostate cancers examined in the study, including those that were poorly differentiated, stained positive for IL-6R [56]. One possible mechanism to account for this observation is that IL-6 affects prostatic growth and function in an autocrine and paracrine manner.

3.3. Effects of IL-6 on human prostate cancer cell lines

Studies of IL-6 expression in prostate cancer were initially carried out in a variety of prostate cancer cell lines. The androgen-refractory cell lines PC-3, DU145, and TSU secrete a number of cytokines including high levels of IL-6 [57]. IL-6 was also detected in the supernatants of cultured prostatic stromal and epithelial cells [50,56,58]. There have been conflicting reports as to whether or not the androgen-responsive cell line LNCaP secretes IL-6. We and others have observed IL-6 production in this cell line [4,54] while other investigators have reported minimal or no IL-6 production by LNCaP cells [57,59]. We have observed that phorbol esters induce IL-6 production from LNCaP cells [60]. Okamoto et al. showed that the addition of dihydrotestosterone (DHT) to the culture medium of LNCaP cells could stimulate the production of IL-6 in a dose-dependent manner [59]. In contrast, we have demonstrated that DHT inhibits phorbol-ester-induced IL-6 production from LNCaP cells [60]. These results are in agreement with other investigators that have demonstrated androgens inhibit IL-6 production in bone marrow stromal cells [61].

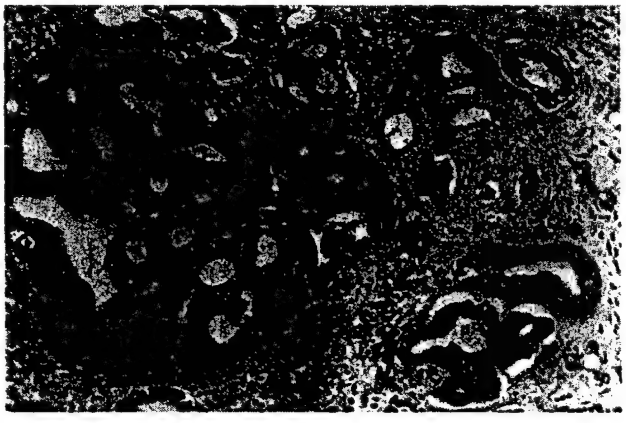

Fig. 1. Immunohistochemical staining for IL-6 receptor on paraffin-embedded adenocarcinoma tissue. The majority of cells express IL-6 receptor (reproduced from Hobisch et al. [56] with permission of John Wiley and Sons).

In contrast to the conflicting data regarding IL-6, the presence of IL-6 receptor is more consistent. Specifically, the hormone refractory cell lines DU-145, PC-3 and TSU, and the hormone-dependent cell lines LNCaP, LNCaP-ATCC, and LNCaP-GW have been shown to express both components of the IL-6 receptor complex. The presence of IL-6 receptor in these cells was first demonstrated with the use of a chimeric toxin, composed of IL-6 and Pseudomonas endotoxin that selectively bound to IL-6 receptor and resulted in cell death [54]. Further studies identified the ligand binding subunit (IL6Rp80) by slot blot analysis [55], ELISA and RT-PCR [57], and the signal transduction subunit (gp130) by sequential immunoprecipitation and immunoblotting [57]. Proliferation studies carried out with prostate cancer cell lines revealed different effects of IL-6 on androgen-sensitive and insensitive cells [57]. Just as there have been contrasting results regarding the production of IL-6 by LNCaP cells, a number of studies from various laboratories have yielded contrasting results regarding the effects of IL-6 on the growth of these cells. Chung et al. showed that inhibition of IL-6 resulted in decreased cell growth of hormone-refractory cells, but had no effect on the growth of hormone-dependent cell lines [57]. Addition of exogenous IL-6 to the culture media of LNCaP cells by several groups has resulted in a dose-dependent inhibition of cell growth [57,58,62–65]. On the other hand, some researchers observed a stimulatoy response after treatment with IL-6 [66,67]. The reasons for these differences have not been clarified to date but it seems that IL-6 in human prostate cancers exerts divergent effects and therefore it will be interesting to learn more about its co-localization with molecules that regulate cellular proliferation. It thus appears that IL-6 acts as an autocrine and paracrine growth factor in PC-3, TSU, and DU145 cells [57], and as a paracrine growth inhibitor in LNCaP cells [57,58]. However, in the presence of androgen, IL-6 acts as an autocrine growth

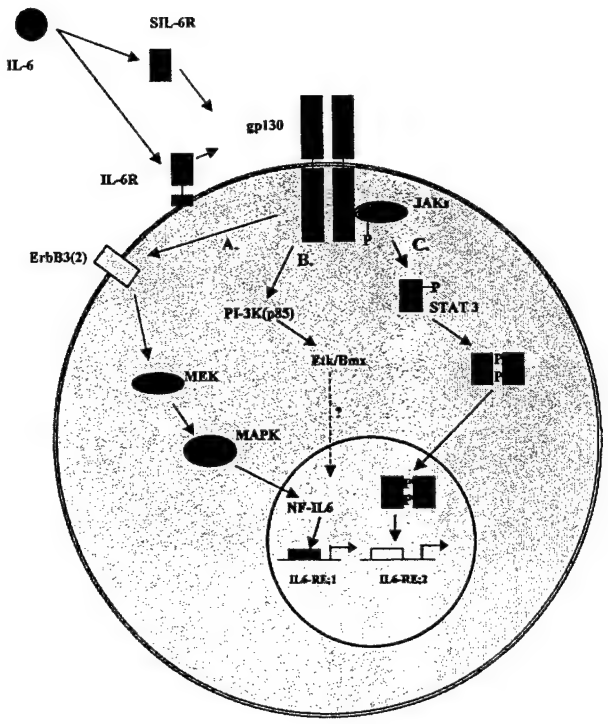

Fig. 2. IL-6 signaling in prostate cancer cells. IL-6 signaling through gp130 initiates three pathways proposed to occur in prostate cancer. (A) IL-6-induced association of gp130 and ErbB3 or ErbB2 leads to activation of signaling through the MAP kinase pathway with subsequent activation of NF-IL-6. Once activated, NF-IL-6 binds to IL-6 response element type one (IL6-RE;1) resulting in gene transcription, (B) IL-6 promotes co-precipitation of gp130 with the p85 subunit of PI3-kinase (PI3-K). PI3-K is involved in Etk/Bmx activation, which leads to nuclear translocation of unidentified transcription factors that activate transcription, (C) IL-6 signaling is primarily mediated through the activation of the JAK/STAT pathway. Homodimerization of gp-130 (following IL-6/IL-6R binding) activates associated tyrosine kinases that subsequently cause gp130 phosphorylation (P). Activated JAK phosphorylates STAT3 which homodimerizes and translocates to the nucleus. Once in the nucleus, this STAT3 homodimer binds to the type 2 IL-6 response element (IL6-RE;2) which initiates transcription.

factor in LNCaP cells [59]. Applying these findings to behavior of prostate cancer in vivo, Chung et al. have suggested that IL-6 may undergo a functional transition from paracrine growth inhibitor to autocrine growth stimulator during the progression of CaP to the hormone refractory phenotype [57].

3.4. IL-6 signaling in prostate cancer

IL-6 mediates its affects on prostate cancer cells through a variety of signal transduction pathways (summarized in Fig. 2). For example, IL-6 activates the PI3-kinase cascade in prostate cancer cells, resulting in differentiation [68] and inhibition of apoptosis [69]. Qiu et al. demonstrated that PI3-kinase is involved in Etk activation based on the following findings: (i) Wortmannin, a specific inhibitor or PI3-kinase, abolished the activation of Etk by IL-6, (ii) a constituitively active p110 subunit of PI3-kinase was able to activate Etk in the absence of IL-6, and (iii) a dominant negative p85 subunit of PI3-kinase mutant blocked the activation of Etk by IL-6 [68]. Consequently, IL-6 treatment of LNCaP induced a neuroendocrine-like differentiation phenotype, with neurite extension and enhanced expression of neuronal markers. The phenotype could be abrogated by the overexpression of a dominant-negative Etk, indicating Etk is required for this differentiation process. In Chung et al.'s study [69], tyrosine phosphorylation of p85 was upregulated by IL-6 in both LNCaP and PC-3 cells. IL-6 promoted co-precipitation of p85 with gp130. Inhibition of PI3-kinase with wortmannin induced programmed cell death in PC-3 cells. Both lines of investigation indicated the participation of PI3-kinase in the IL-6 pathway. Qiu et al. also discovered that binding of IL-6 in LNCaP cells induced the association of gp130 with growth factor receptors (ErbB2, ErbB3), and subsequently leads to activation of the MAP kinase pathway [66].

In general, IL-6 signaling is primarily mediated through the activation of JAK/STAT pathway. Lou et al. showed that IL-6 stimulates prostate cancer growth through this pathway [70]. Another study reports that STAT3 transcriptional activation correlates with the growth-inhibitory signal of IL-6 in LNCaP cells, suggesting that STAT3 transcriptional activity is an important determinant in the different phenotypic responses to IL-6 in prostate cancer [71]. Moreover, Chen et al. reported that IL-6-induced activation of STAT3 in LNCaP cells increased AR-mediated gene activation. In particular, they showed that STAT3 associated with the AR in an androgen-independent, but IL-6 dependent, manner [72]. This reveals the importance of IL-6 signaling to induce AR-mediated gene activation in prostate carcinoma cells and the importance of activated STAT3 in human tumor development and progression.

3.5. IL-6 and the androgen receptor

The AR, which is expressed in normal prostate tissue and heterogeneously in prostate cancers, is a key transcription factor in the prostate [73–76]. Activation of the AR in prostate cancer is being intensively investigated, and there is evidence that the AR could be stimulated by a number of nonsteroidal compounds, such as polypeptide growth factors, protein kinase A activators, vitamin D and neuropeptides [77–81]. IL-6 activates the AR in a ligand-independent manner and induces a synergistic AR response with very low concentrations of androgen [62,72]. Our previous report that androgen inhibits AR activity [60] combined with the observation that IL-6 activates AR demonstrates that cross talk between IL-6 and AR exists. The interaction between IL-6 and AR might be particularly

important in patients with advanced prostate cancer who have elevated serum levels of IL-6 [48–50].

3.6. IL-6 and chemotherapy

Defining mechanisms to control IL-6 or IL-6R expression may prove useful for therapy of the many clinical disorders in which IL-6 plays a role [4]. Addition of anti-IL-6 antibody to the growth medium of the hormone independent cell lines DU145, PC-3 [57,82], and TSU [57] inhibits cell growth. In vitro studies have shown that the addition of anti-IL-6 enhances the cytotoxicity of certain chemotherapeutic agents in PC-3 cells, which are resistant to the drugs alone [82]. Borsellino et al. later showed that the activity of IL-6 was more efficiently blocked with an IL-6R antagonist, Sant7, and that this too potentiated the sensitivity of PC-3 cells to etoposide-mediated cytotoxicity [83]. In this study, the investigators also blocked signaling through gp130 in PC-3 using a gp130 antisense oligodeoxynucleotide. This inhibited cell growth and viability by about 20% and increased sensitivity to etoposide, confirming the positive role of endogenous IL-6 in cell survival [83]. Furthermore, Borsellino et al. showed that gp130-mediated signaling does not influence or minimally influences the bcl-2 mediated antiapoptotic pathway and that, rather, it proceeds through a *ras*-dependent pathway [83]. These data suggest that endogenous IL-6 acts to protect tumor cells from drug-induced cell death, and its neutralization may be a useful adjuvant to chemotherapy.

4. Conclusion

Clinical studies of prostate cancer patients have implicated IL-6 as a potential mediator of prostate cancer morbidity and as a marker for advanced hormone-refractory prostate cancer. The various functions of IL-6 may allow it to play multiple roles in the pathophysiology of prostate cancer. It has been shown to act as an autocrine and paracrine growth factor in hormone refractory human prostate cancer cell lines and as a paracrine growth inhibitor in hormone dependent cell lines. These data suggest that IL-6 plays a role in the transition of prostate cancer from a hormone-dependent to a hormone-refractory phenotype. The ability of IL-6 to mediate signaling through the AR in the absence of androgen also lends credence to this hypothesis. In vitro studies have also shown that IL-6 plays a key role in protecting prostate cancer cells from chemotherapeutic-mediated cell death. Some of the signaling pathways through which IL-6 mediates its effects have been defined, giving us a better understanding of how IL-6 contributes to the pathophysiology of the disease. Taken together, these studies suggest that the inhibition of IL-6 may be a useful adjunct to prostate cancer therapy as in vitro data have shown, and could decrease morbidity in patients with advanced disease.

Acknowledgements

This work was supported by USAMRMC Prostate Cancer Research Program Grant # DAMD17-00-1-053, National Institutes of Health Grants SPORE 1 P50 CA69568, T32 RR07008, and R01 AG039738.

References

[1] Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics 1999. CA Cancer J Clin 1999;49:8–31.
[2] Hegarty NJ, Fitzpatrick JM, Richie JP, Scardino PT, deVere White RW, Schroder FH, et al. Future prospects in prostate cancer. Prostate 1999;40:261–8.
[3] Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 1999;5:280–5.
[4] Keller ET, Wanagat J, Ershler WB. Molecular and cellular biology of interleukin-6 and its receptor. Front Biosci 1996;1:340–57.
[5] May LT, Ghrayeb J, Santhanam U, Tatter SB, Sthoeger Z, Helfgott DC, et al. Synthesis and secretion of multiple forms of beta 2-interferon/B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes. J Biol Chem 1988;263:7760–6.
[6] Sehgal P, Wang L, Rayanade R, Pan H, Margulies L. Interleukin-6 type Cytokines. In: Mackiewicz A, Koji A, Sehgal P, editors. Annals New York Academy of Science. New York: New York Academy of Sciences, 1995:1–14.
[7] Hirano T. The biology of interleukin-6. Chem Immunol 1992;51:153–80.
[8] Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, et al. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 1989;58:573–81.
[9] Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997;15:797–819.
[10] Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, et al. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science 1988;241:825–8.
[11] Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T. Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell 1990;63:1149–57.
[12] Ward LD, Howlett GJ, Discolo G, Yasukawa K, Hammacher A, Moritz RL, et al. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. J Biol Chem 1994;269:23286–9.
[13] Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, et al. IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 1993;260:1808–10.
[14] Fukada T, Hibi M, Yamanaka Y, Takahashi-Tezuka M, Fujitani Y, Yamaguchi T, et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 1996;5:449–60.
[15] Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, et al. Association and activation of Jak-Tyk kinases by

CNTF-LIF-OSM-IL-6 beta receptor components. Science 1994;263:92–5.
[16] Ernst M, Gearing DP, Dunn AR. Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells. Embo J 1994;13:1574–84.
[17] Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Jr., Yancopoulos GD. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science 1995;267:1349–53.
[18] Yamanaka Y, Nakajima K, Fukada T, Hibi M, Hirano T. Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. Embo J 1996;15:1557–65.
[19] Tomida M, Heike T, Yokota T. Cytoplasmic domains of the leukemia inhibitory factor receptor required for STAT3 activation, differentiation, and growth arrest of myeloid leukemic cells. Blood 1999;93:1934–41.
[20] Mullberg J, Oberthur W, Lottspeich F, Mehl E, Dittrich E, Graeve L, et al. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. J Immunol 1994;152:4958–68.
[21] Rose-John S, Heinrich PC. Soluble receptors for cytokines and growth factors: generation and biological function. Biochem J 1994;300:281–90.
[22] Lust JA, Donovan KA, Kline MP, Greipp PR, Kyle RA, Maihle NJ. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. Cytokine 1992;4:96–100.
[23] Holub MC, Szalai C, Polgar A, Toth S, Falus A. Generation of 'truncated' interleukin-6 receptor (IL-6R) mRNA by alternative splicing; a possible source of soluble IL-6R. Immunol Lett 1999;68:121–4.
[24] Horiuchi S, Ampofo W, Koyanagi Y, Yamashita A, Waki M, Matsumoto A, et al. High-level production of alternatively spliced soluble interleukin-6 receptor in serum of patients with adult T-cell leukaemia/HTLV-I-associated myelopathy. Immunology 1998;95:360–9.
[25] Saily M, Koistinen P, Pulkki K, Zheng A, Savolainen ER. Acute myeloblastic leukaemia cells produce soluble interleukin 6 receptor by a mechanism of alternative splicing. Cytokine 1998;10:860–7.
[26] Mackiewicz A, Schooltink H, Heinrich PC, Rose-John S. Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins. J Immunol 1992;149:2021–7.
[27] Guschin D, Rogers N, Briscoe J, Witthuhn B, Watling D, Horn F, et al. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. Embo J 1995;14:1421–9.
[28] Schindler C, Darnell JE, Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 1995;64:621–51.
[29] Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91–related transcription factor involved in the gp130-mediated signaling pathway. Cell 1994;77:63–71.
[30] Fourcin M, Chevalier S, Guillet C, Robledo O, Froger J, Pouplard-Barthelaix A, et al. Gp130 transducing receptor cross-linking is sufficient to induce interleukin-6 type responses. J Biol Chem 1996;271:11756–60.
[31] Giordano V, De Falco G, Chiari R, Quinto I, Pelicci PG, Bartholomew L, et al. Shc mediates IL-6 signaling by interacting with gp130 and Jak2 kinase. J Immunol 1997;158:4097–103.
[32] Lai CF, Ripperger J, Morella KK, Wang Y, Gearing DP, Horseman ND, et al. STAT3 and STAT5B are targets of two different signal pathways activated by hematopoietin receptors and control transcription via separate cytokine response elements. J Biol Chem 1995;270:23254–7.
[33] Sadowski HB, Shuai K, Darnell JE, Jr., Gilman MZ. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 1993;261:1739–44.
[34] Yuan J, Wegenka UM, Lutticken C, Buschmann J, Decker T, Schindler C, et al. The signalling pathways of interleukin-6 and gamma interferon converge by the activation of different transcription factors which bind to common responsive DNA elements. Mol Cell Biol 1994;14:1657–68.
[35] Zhong Z, Wen Z, Darnell JE, Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994;264:95–8.
[36] Wen Z, Zhong Z, Darnell JE, Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241–50.
[37] Schuringa JJ, Jonk LJ, Dokter WH, Vellenga E, Kruijer W. Interleukin-6-induced STAT3 transactivation and ser727 phosphorylation involves vav, rac-1 and the kinase SEK-1/MKK-4 as signal transduction components. Biochem J 2000;347(1):89–96.
[38] Chen-Kiang S. Regulation of terminal differentiation of human B-cells by IL-6. Curr Top Microbiol Immunol 1995;194:189–98.
[39] Ernst M, Oates A, Dunn AR. Gp130-mediated signal transduction in embryonic stem cells involves activation of Jak and Ras/mitogen-activated protein kinase pathways. J Biol Chem 1996;271:30136–43.
[40] Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253–62.
[41] Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. Science 1995;268:251–5.
[42] Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, et al. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. Embo J 1990;9:1897–906.
[43] Matsumoto M, Sakao Y, Akira S. Inducible expression of nuclear factor IL-6 increases endogenous gene expression of macrophage inflammatory protein-1 alpha, osteopontin and CD14 in a monocytic leukemia cell line. Int Immunol 1998;10:1825–35.
[44] Yin T, Yang YC. Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in signaling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor, and oncostatin M in mouse 3T3-L1 cells. J Biol Chem 1994;269:3731–8.
[45] Boulton TG, Stahl N, Yancopoulos GD. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J Biol Chem 1994;269:11648–55.
[46] Chen RH, Chang MC, Su YH, Tsai YT, Kuo ML. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J Biol Chem 1999;274:23013–9.
[47] Takahashi-Tezuka M, Hibi M, Fujitani Y, Fukada T, Yamaguchi T, Hirano T. Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. Oncogene 1997;14:2273–82.
[48] Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC. Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. J Urol 1999;161:182–7.
[49] Hoosein N, Abdul M, McCabe R, Gero E, Deftos L, Banks M, et al. Clinical significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostatic carcinoma. Urol Oncol 1995;1:246–51.
[50] Twillie DA, Eisenberger MA, Carducci MA, Hseih WS, Kim WY, Simons JW. Interleukin-6: a candidate mediator of human prostate cancer morbidity. Urology 1995;45:542–9.

[51] Drachenberg DE, Elgamal AA, Rowbotham R, Peterson M, Murphy GP. Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. Prostate 1999;41:127–33.
[52] Hanks G, Meyers C, Scardino P. Cancer of the prostate. In: Devita VJ, Hellman S, Rosenberg S, editors. Cancer: principles and practice of oncology, 4th ed. Philadelphia: Lippincott, 1993:1073–113.
[53] Strassmann G, Fong M, Kenney JS, Jacob CO. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest 1992;89:1681–4.
[54] Siegall CB, Schwab G, Nordan RP, FitzGerald DJ, Pastan I. Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. Cancer Res 1990;50:7786–8.
[55] Siegsmund MJ, Yamazaki H, Pastan I. Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia. J Urol 1994;151:1396–9.
[56] Hobisch A, Rogatsch H, Hittmair A, Fuchs D, Bartsch G, Jr., Klocker H, et al. Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. J Pathol 2000;191:239–44.
[57] Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role of IL-6 in the progression of prostate cancer. Prostate 1999;38:199–207.
[58] Degeorges A, Tatoud R, Fauvel-Lafeve F, Podgorniak MP, Millot G, de Cremoux P, et al. Stromal cells from human benign prostate hyperplasia produce a growth-inhibitory factor for LNCaP prostate cancer cells, identified as interleukin-6. Int J Cancer 1996;68:207–14.
[59] Okamoto M, Lee C, Oyasu R. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. Cancer Res 1997;57:141–6.
[60] Keller ET, Chang C, Ershler WB. Inhibition of NFkappaB activity through maintenance of IkappaBalpha levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J Biol Chem 1996;271:26267–75.
[61] Bellido T, Jilka RL, Boyce BF, Girasole G, Broxmeyer H, Dalrymple SA, et al. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. J Clin Invest 1995;95:2886–95.
[62] Hobisch A, Eder IE, Putz T, Horninger W, Bartsch G, Klocker H, et al. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res 1998;58:4640–5.
[63] Levesque E, Beaulieu M, Guillemette C, Hum DW, Belanger A. Effect of interleukins on UGT2B15 and UGT2B17 steroid uridine diphosphate-glucuronosyltransferase expression and activity in the LNCaP cell line. Endocrinology 1998;139:2375–81.
[64] Mori S, Murakami-Mori K, Bonavida B. Dexamethasone enhances expression of membrane and soluble interleukin-6 receptors by prostate carcinoma cell lines. Anticancer Res 1998;18:4403–8.
[65] Ritchie CK, Andrews LR, Thomas KG, Tindall DJ, Fitzpatrick LA. The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. Endocrinology 1997;138:1145–50.
[66] Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. Nature 1998;393:83–5.
[67] Okamoto M, Lee C, Oyasu R. Autocrine effect of androgen on proliferation of an androgen responsive prostatic carcinoma cell line, LNCAP: role of interleukin-6. Endocrinology 1997;138:5071–4.
[68] Qiu Y, Robinson D, Pretlow TG, Kung HJ. Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc Natl Acad Sci USA 1998;95:3644–9.
[69] Chung TD, Yu JJ, Kong TA, Spiotto MT, Lin JM. Interleukin-6 activates phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. Prostate 2000;42:1–7.
[70] Lou W, Ni Z, Dyer K, Tweardy DJ, Gao AC. Interleukin-6 induces prostate cancer cell growth accompanied by activation of stat3 signaling pathway. Prostate 2000;42:239–42.
[71] Spiotto MT, Chung TD. STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. Prostate 2000;42:186–95.
[72] Chen T, Wang LH, Farrar WL. Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. Cancer Res 2000;60:2132–5.
[73] Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995;55:3068–72.
[74] Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Androgen receptor status of lymph node metastases from prostate cancer. Prostate 1996;28:129–35.
[75] Sadi MV, Walsh PC, Barrack ER. Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy. Cancer 1991;67:3057–64.
[76] Van der Kwast THS, Ruizeveld de Winter JA, van Vroonhoven CCJ, Mulder E, Boersma W, Trapman J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 1991;48:189–93.
[77] Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474–8.
[78] Culig Z, Hobisch A, Hittmair A, Cronauer MV, Radmayr C, Zhang J, et al. Synergistic activation of androgen receptor by androgen and luteinizing hormone-releasing hormone in prostatic carcinoma cells. Prostate 1997;32:106–14.
[79] Nakhla AM, Romas NA, Rosner W. Estradiol activates the prostate androgen receptor and prostate-specific antigen secretion through the intermediacy of sex hormone-binding globulin. J Biol Chem 1997;272:6838–41.
[80] Nazareth LV, Weigel NL. Activation of the human androgen receptor through a protein kinase A signaling pathway. J Biol Chem 1996;271:19900–7.
[81] Zhao XY, Ly LH, Peehl DM, Feldman D. 1alpha,25-dihydroxyvitamin D3 actions in LNCaP human prostate cancer cells are androgen-dependent. Endocrinology 1997;138:3290–8.
[82] Borsellino N, Belldegrun A, Bonavida B. Endogenous interleukin 6 is a resistance factor for *cis*-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. Cancer Res 1995;55:4633–9.
[83] Borsellino N, Bonavida B, Ciliberto G, Toniatti C, Travali S, D'Alessandro N. Blocking signaling through the Gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumor cells to etoposide and cisplatin-mediated cytotoxicity. Cancer 1999;85:134–44.

Cancer and Metastasis Reviews **20:** 333–349, 2001.

Prostate carcinoma skeletal metastases: Cross-talk between tumor and bone

Evan T. Keller[1], Jian Zhang[1], Carlton R. Cooper[2], Peter C. Smith[1], Laurie K. McCauley[3], Kenneth J. Pienta[2] and Russell S. Taichman[3]
[1]*Unit for Laboratory Animal Medicine,* [2]*Department of Surgery,* [3]*Department of Periodontics/Prevention/Geriatrics, University of Michigan, Ann Arbor, MI, USA*

Key words: prostatic neoplasms, skeletal metastases, bone morphogenetic protein, parathyroid hormone-related protein, stromal-derived factor, matrix metalloproteinase

Abstract

The majority of men with progressive prostate cancer develop metastases with the skeleton being the most prevalent metastatic site. Unlike many other tumors that metastasize to bone and form osteolytic lesions, prostate carcinomas form osteoblastic lesions. However, histological evaluation of these lesions reveals the presence of underlying osteoclastic activity. These lesions are painful, resulting in diminished quality of life of the patient. There is emerging evidence that prostate carcinomas establish and thrive in the skeleton due to cross-talk between the bone microenvironment and tumor cells. Bone provides chemotactic factors, adhesion factors, and growth factors that allow the prostate carcinoma cells to target and proliferate in the skeleton. The prostate carcinoma cells reciprocate through production of osteoblastic and osteolytic factors that modulate bone remodeling. The prostate carcinoma-induced osteolysis promotes release of the many growth factors within the bone extracellular matrix thus further enhancing the progression of the metastases. This review focuses on the interaction between the bone and the prostate carcinoma cells that allow for development and progression of prostate carcinoma skeletal metastases.

1. Introduction

Prostate carcinoma is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States [1]. The most common site of prostate carcinoma metastasis is the bone with skeletal metastases identified at autopsy in up to 90% of patients dying from prostate carcinoma [2–4]. Skeletal metastasis results in significant complications that diminish the quality of life in affected patients. These complications include bone pain, impaired mobility, pathological fracture, spinal cord compression and symptomatic hypercalcemia [5–7]. Despite advances in the diagnosis and management of prostate carcinoma, advanced disease with skeletal metastasis remains incurable. Current therapeutic modalities are mostly palliative, and include hormonal therapy, pharmacological management of bone pain, radiotherapy for pain and spinal cord compression [8], various chemotherapy regimens, and the use of bisphosphonates to inhibit osteoclast activity [9]. In spite of the severe complications of prostate carcinoma skeletal metastasis, there have not been many advances in the therapeutic arena to prevent or diminish these lesions. It is critical that a solid understanding of the pathophysiology of prostate carcinoma skeletal metastatic process is developed to provide the basis for creating strategies to prevent or diminish their occurrence and associated complications. A preponderance of evidence suggests that establishment and progression of prostate carcinoma bone metastases is dependent on interaction between the bone microenvironment and the prostate carcinoma cell through both soluble and cell-membrane bound bioactive factors. In this review, we will summarize some of the cross-talk mechanisms between bone and prostate carcinoma.

2. The effects of bone on prostate carcinoma metastasis

In agreement with the 'seed and soil' theory of metastases espoused by Paget [10], the predilection of prostate carcinoma to establish metastases in bone as

opposed to other organs suggests that the bone microenvironment offers a fertile soil for prostate carcinoma growth. Prior to interacting on the bone cells and bone matrix, the prostate carcinoma cells must enter the bone compartment. This is accomplished by several general mechanisms that include chemotaxis from the circulation, attachment to bone endothelium, extravasation, and invasion. The bone microenvironment is a complicated mixture of mineralized and non-mineralized bone matrix and endothelial, hematopoietic, immune, and bone marrow stromal cells. Each of these components of the bone microenvironment may contribute to the establishment of prostate carcinoma metastases through provision of chemotactic, angiogenic, adhesion and growth factors.

2.1. Chemotaxis

When prostate carcinoma cells are injected adjacent to adult human bone implanted in SCID mice, the prostate carcinoma cells to migrate to adult human bone [11]. This observation provides evidence that bone provides chemotactic factors for prostate carcinoma cells. This is further supported by the observation that bone undergoing active resorption facilitated adhesion [12] and chemotaxis [13,14] of tumor cells to bone compared to non-resorbing bone. Collagen products appear to be one component of bone that induces tumor chemotaxis [15]. The factors through which bone induces chemotaxis are not clear. However, low glycosylated osteonectin was found to be an active chemotaxic factor in crude bone extracts that promoted chemotaxis of human prostate epithelial cells and increased the invasive ability of human prostate carcinoma cells [16]. In contrast with this observation, purified fibronectin, but not crude bone extracts induced migration of the prostate carcinoma DU-145 cell line [17]. Cell line specificity may account for these differences. Epidermal growth factor induced migration of the TSU-pr1 prostate carcinoma cell line [18]. Since EGF is present in medullary bone, this observation suggests that it may act as a chemotactic factor for bone metastases. Finally, the Rho-kinase inhibitor, Y-27632, inhibited *in vitro* chemotactic migration to bone marrow fibroblast conditioned media and metastatic growth in immune-compromised mice of highly invasive human prostatic cancer (PC3) cells [19]. This observation suggests that modulation of kinase activity may prove fruitful in inhibition of skeletal metastasis.

In addition to the above substances, which typically are not considered chemotactic factors, prostate carcinoma cells may commandeer the normal leukocyte bone marrow homing mechanism using the chemokine pathway [20]. Chemokines are classified based upon the relative position of cysteine residues near the NH2-terminus into four major families: CC,CXC,C,CX_3C (as reviewed in [21]). Chemokines activate receptors that are members of the large family of seven-transmembrane G protein–coupled proteins. In addition to the role that chemokines have in cell migration, they play significant roles in normal development, inflammation, atherosclerosis and angiogenesis. The rapidly increasing knowledge of chemokines has begun to impact many aspects of tumor biology including modulation of proliferation, angiogenesis and immune response to tumor (as reviewed in [22]).

An important role for chemokines may be to regulate metastatic behavior. Localization in tissues and migration to target organs are essential steps in the pathobiology of metastasis which strongly support the analogy to hematopoietic cell homing. In this context, the CXC chemokine stromal-derived factor (SDF-1; CXCL12) and its receptor, CXCR4 appear to be critical molecular determinants for these events [23,24]. This has been substantiated in gene knockout investigations [25,26] and by the demonstration that level of CXCR4 expression correlates with the ability of human hematopoietic progenitors to engraft into nude mice [26]. In the bone marrow, SDF-1 is constitutively produced by osteoblasts, fibroblasts and endothelial cells [27]. However, not all vascular endothelial cells express SDF-1, suggesting that organ-specific expression SDF-1 may account for the selectivity of metastases to target certain organs [28].

Several lines of evidence suggest that SDF-1 contributes to the pathogenesis of prostate carcinoma metastases. Inhibition of chemokines diminished *in vitro* proliferation of PC-3 cells [29] and anti-CXCR2 antibody inhibited IL-8-stimulated migration of PC-3 cells *in vitro* [30]. These studies suggest that chemokines contribute to prostate metastatic pathophysiology. This possibility is reinforced by the observation that CXCR4 is expressed in normal prostate tissues, albeit at low levels [31], as well as several neoplasms that invade the marrow (e.g., breast cancers, Burkitt's lymphoma, leukemias) [31–33]. Furthermore, several prostate carcinoma cell lines express CXCR4 mRNA, and SDF-1 increased migration of these cells *in vitro* [34]. It was recently demonstrated that normal breast tissues express little CXCR4, whereas breast neoplasms express high levels of CXCR4 [35,36], and antibody to CXCR4 blocked the

metastatic spread of the tumors to the bone in an experimental metastasis model [35]. Taken together, these data suggest that SDF-1 and CXCR4 are likely critical regulators of prostate carcinoma metastasis to bone.

2.2. *Attachment to endothelium*

Cell adhesion plays a vital role in cancer metastasis. In fact, the ability of cancer cells to adhere to organ-specific cells and components may be a critical regulator of their metastatic pattern. A cancer cell in the circulation initially interacts with the organ's microvascular endothelium and subsequently the organ's extracellular matrix (ECM) components [37,38]. Cell adhesion molecules (CAMs) expressed on both the cancer and endothelial cells mediate these interactions. CAMs expressed on the endothelial cells are regulated by an organ's microenvironment, which results in CAM expression specific to each organ [39]. The organ-specific composition of ECM proteins such as laminin, fibronectin, and vitronectin that are recognized by CAMs expressed on cancer cells contribute significantly to organ-specific metastasis [40,41].

It has been proposed that prostate carcinoma metastasis to bone is mediated, in part, by preferential adhesion to bone marrow endothelium as opposed to endothelium from other sites [42,43]. Two studies demonstrated that prostate carcinoma cells adhered preferentially to immortalized human bone marrow endothelial (HBME) cells as compared to human umbilical vein endothelial cells (HUVEC), immortalized human aortic endothelial cells (HAEC-I), and immortalized human dermal microvascular endothelial cells (HDMVEC) [42,44]. This observation was confirmed in another study that demonstrated preferential adhesion of PC-3 cells to HBME cells as compared to HUVECs and lung endothelial cells, Hs888Lu [45]. Interestingly, this adhesion was enhanced when HBME cells were grown on bone ECM components [44]. The PC-3 cell line was used as a model for prostate carcinoma in these studies because it was derived from a bone metastasis. To determine the CAMs involved in prostate carcinoma (PC)–HBME interaction, galactose-rich-modified citrus pectin (MCP) and several antibodies to known CAMs expressed on HBME cell monolayers, were used in adhesion assays. MCP was used because it was reported to interfere with interactions mediated by carbohydrate-binding proteins such as galectins [46]. The data demonstrated that MCP and antibodies to galectin-3, vascular cell adhesion molecule (VCAM), CD11a (alpha-L), CD18 (beta-2), and leukocyte functional antigen-1 (LFA-1) pectin, reduced PC-3 cell adhesion to HBME cell monolayers [42]. This observation suggests that carbohydrate-binding proteins, VCAM, alpha-L, beta-2, and LFA-1 may be partially involved in prostate carcinoma cell adhesion to HBME cells. Beta-1 integrins expressed on HUVEC were demonstrated to mediate PC-3 cell adhesion to this endothelial cell line [47]. Surprisingly, the beta-1 integrins expressed on HBME cells were not involved in PC-3 cell adhesion to HBME cell monolayers [48]; however, beta-1 integrins, expressed on PC-3 cells, did mediate its interaction with HBME cell monolayers [45]. Hyaluronan and galactosyl receptor, a cell surface C-type lectin expressed on PC-3 cells, were also shown to mediate PC–HBME interaction [49,50].

The ability of metastatic prostate cells to adhere to the bone matrix may also contribute to prostate carcinoma frequent metastasis to bone matrix [51,52]. Kostenuik demonstrated that PC-3 cells adhered to the collagen type I in the bone matrix. This adhesion was mediated by $\alpha 2\beta 1$ expressed on PC-3 cells and was upregulated by transforming growth factor-β (TGF-β), a major bone-derived cytokine [53]. Festuccia and colleagues [52] showed that osteoblast-conditioned media containing TGF-β, modulated the PC-3 interaction with ECM proteins, including collagen type I. These results provide evidence that TGF-β, present in the bone marrow, can influence prostate carcinoma cell adhesion to the bone matrix by modulating surface expression of selected integrins.

2.3. *Growth factors*

The calcified bone matrix is replete with putative prostate carcinoma growth factors including insulin-like growth factors (IGF), bone morphogenetic proteins (BMP), fibroblast growth factors (FGF) and transforming growth factor (TGF)-beta, which are released upon resorption of bone [54,55]. Furthermore, experimental evidence that resorption of calcified bone matrix promotes tumor growth was suggested by the observation that conditioned media for bone cultures undergoing resorption stimulated cancer cell growth of a variety of tumor cell lines [56]. Taken together, these data suggest that inhibiting bone resorption will diminish cancer growth by decreasing growth factors availability in the bone microenvironment.

Several purified factors from bone matrix have been demonstrated to stimulate prostate carcinoma cell growth *in vitro* [57–59]. For example, IGF-I

and IGF-II are important mediators of prostate carcinoma growth (as reviewed in [60,61]). Prostate carcinoma cells have IGF receptors [62] and proliferate in response to IGF [57]. Transfection of LNCaP cells with FGF-8 expression vector induced an increased growth rate, higher soft agar clonogenic efficiency, enhanced *in vitro* invasion, and increased *in vivo* tumorigenesis [58]. The source of these growth factors is diverse. For example, osteoblast-derived factors influence prostate carcinoma growth, adhesion, and motility [16,17,63]. Additionally, bone marrow stromal cells, as opposed to non-skeletal fibroblasts, induced prostate carcinoma cell growth *in vitro* and *in vivo* [64–66]. As research continues on the extracellular matrix of bone, it is very likely that additional prostate carcinoma growth factors will be discovered.

3. The effect of prostate carcinoma on the bone: Osteoblastic

3.1. Prostate skeletal metastases are mixed osteoblastic and osteolytic lesions

Once in the bone, prostate carcinoma tumors have pathobiology that appears to be somewhat unique to cancer skeletal metastases. Specifically, prostate carcinoma skeletal metastases are most often characterized as osteoblastic (i.e., increased mineral density at the site of the lesion) as opposed to osteolytic. Other tumors, such as breast cancer, can form osteoblastic lesions; however, these occur less frequently [67,68]. In spite of the radiographic osteoblastic appearance it is clear from histological evidence that prostate carcinoma metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions although osteoblastic lesions predominate [69–72]. Sites of prostate carcinoma bone metastases are often demonstrated to have increases in osteoid surface, osteoid volume, mineralization rates [73,74]. Recent evidence shows that osteoblastic metastases form on trabecular bone at sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation [75,76]. Clinical evidence demonstrates increased systemic markers of both bone production and bone resorption in prostate carcinoma patients [77,78] in addition to bone histomorphometric findings of increased indices of bone resorption [71]. These findings suggest that prostate carcinoma induces bone production through an overall increase in bone remodeling, which in the non-pathologic state is a balance between osteoclastic resorption of bone and osteoblast-mediated replacement of resorbed bone (as reviewed in [79–81]). In the case of prostate carcinoma, it appears the induction of osteoblast-mediated mineralization outweighs the increase in osteoclast resorption resulting in overall formation of osteoblastic lesions. The osteoblastic lesions result in overall weakening of the bone for the following reasons; mature, healthy bone is formed of lamellar bone, which allows for tight packing of collagen bundles and optimum bone strength. In contrast, prostate carcinoma induces production of *woven* bone, which is composed of loosely packed, randomly oriented collagen bundles that produce bone with suboptimal strength [82,83]. Thus, the combination of underlying osteolysis and production of weak bone leads to a predisposition to fracture. The mechanisms through which prostate carcinoma cells promote bone mineralization remain poorly understood.

3.2. A variety of factors may contribute to prostate carcinoma-mediated bone mineralization

Prostate carcinoma produces osteoblastic factors that mediate their effect through activation of the osteoblast transcription factor Cbfa1 in the osteoblast precursor [84]. This suggests that induction of osteosclerosis occurs through normal osteoblast differentiation pathways. In addition to this observation, the prostate carcinoma cell itself demonstrates increased expression of Cbfa1 an the ability to mineralize *in vitro*, suggesting that it directly contributes to osteosclerosis [85]. Many factors that have direct or indirect osteogenic properties have been implicated in prostate carcinoma's osteogenic activity (Table 1) (as reviewed in [86, 87–89]). Although, initially identified as a non-defined osteoblastic activity from prostate carcinoma cells *in vitro* [90], many specific factors have been

Table 1. Osteogenic factors produced by cancer cells

Factor	Reference
Bone morphogenetic proteins (BMP)	[93,169]
Endothelin-1 (ET-1)	[94,136]
Insulin-like growth factors (IGF)	[231,232].
Interleukin-1 and -6	[233,234]
Osteoprotegerin (OPG)	[100,101]
Parathyroid hormone-related peptide (PTHrP)	[96,97]
Transforming growth factor-β (TFG-β)	[99]
Urinary plasminogen activator (urokinase)	[235]

identified that may promote osteoblastic lesions. Some of these factors, such as bone morphogenetic proteins (BMP) [91–93] and enodothlin-1 (ET-1) [94] may directly stimulate differentiation of osteoblast precursors to mature mineral-producing osteoblasts [95] or induce osteoblast protein production [93]. Other factors such as parathyroid hormone-related protein (PTHrP) may work through inhibition of osteoblast apoptosis [96,97]. Additionally, there are proteins that may work indirectly to enhance bone production, such as the serine proteases, prostate specific antigen (PSA) and urinary plasminogen activator (uPA), which can activate latent forms of osteogenic proteins, such as transforming growth factor-β (TFG-β [98,99]. Finally, some molecules, such as osteoprotegerin (OPG) [100–102] and ET-1 (in a dual role with its osteoblast-stimulating activity) [103] can enhance osteosclerosis through inhibiting osteoclastogenesis. Other tumor types, such as osteosarcoma, are also known to produce a variety of osteoblastic factors [104–106]. With such a large number of factors, it is difficult to determine which the key factor is, and most likely several of these osteogenic factors work in concert to produce maximal bone production.

3.2.1. Parathyroid hormone related protein (PTHrP)

PTHrP was originally identified as a tumor-derived factor responsible for humoral hypercalcemia of malignancy (HHM). It has limited homology with the endocrine hormone, parathyroid hormone, sharing 7 of the first 13 N-terminal amino acids, but otherwise is dissimilar and immunologically distinct [107]. PTH AND PTHrP bind to the same receptor (the PTH-1 receptor) and evoke the same biological activity due to similarities in their steric configurations at the region of 25–34 amino acids. Patients with solid tumors and hypercalcemia have increased serum PTHrP in 80% of the cases, emphasizing the impact of this peptide to increase bone resorption and renal tubular resorption of calcium [107]. Subsequent to its characterization in HHM, PTHrP was found to be produced by many normal tissues including, epithelium, lactating mammary gland, and cartilage where it has an autocrine, paracrine, or intracrine role [107]. PTHrP plays a critical role in the development of the skeleton as evidenced by its lethality upon gene ablation and the severe skeletal chondrodysplasia found in these animals [108]. These studies have led to the conclusion that PTHrP in cartilage functions to accelerate the growth of cartilage cells and to oppose their progression to a terminally differentiated cell [109].

Many features of PTHrP make it an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid [87,110]. PTHrP has been immunohistochemically identified in prostate carcinoma tissue in patients with clinically localized disease [111], is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium, is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia [112,113], and is found in human metastatic lesions in bone [114]. There is also evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma [115], enhance breast cancer metastasis to bone [116,117], and act as an autocrine growth factor for prostate carcinoma cells *in vitro* [118]. Recent evidence indicates that expression of nuclear-targeted PTHrP can protect prostate and other cells from apoptosis [114,119], bind RNA [120], and act as a mitogen [121,122]. PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model [114] suggesting that PTHrP acts in autocrine or intracrine mechanisms to promote tumor growth. In contrast, in this same model and in an intracardiac injection model of prostate carcinoma, PTHrP was not associated with an increase in metastatic potential [83,114]. This suggests that PTHrP is not important in the process of metastasis to bone but once in the bone microenvironment where target cells with receptors are present (osteoblasts); it may play a critical role in the bone response to prostate carcinoma. Of particular interest to prostate carcinoma, PSA has been shown to cleave PTHrP leading to an inactivation of the PTHrP-stimulation of cAMP which is a key pathway for the actions of PTHrP in bone [123]. More recent studies indicate that in colon cancer cells, PTHrP enhances adhesion of cells to type I collagen but not fibronectin or laminin [124]. All these data suggest that PTHrP has a critical role in the local bone microenvironment of metastatic prostate carcinoma; but what this precise role is has yet to be determined.

3.2.2. Endothelin-1

ET-1 is a member of the ET family which is composed of ET-1, -2, and -3. The ET family members are synthesized as a 203 amino acid precursor peptide that is cleaved to a 21 amino acid peptide with the same two characteristic disulfide bridges [125]. Initially

identified as a potent vasoconstrictor, ET-1 interacts with cell surface ET_A and ET_B receptors to induce a variety of responses including modulation of cell growth and fetal development (as reviewed in [125]). ETs are found in a variety of tissues including vascular endothelium, parathyroid gland, mammary tissue, and macrophages [125].

The role of ET-1 in bone remodeling is controversial. For example, in the murine osteoblast precursor cell, MC3T3-E1, E1 inhibits differentiation, reduces both alkaline phosphatase activity and osteocalcin expression and diminishes *in vitro* mineralization suggesting that ET-1 will diminish bone production [126,127]. In contrast, ET-1 has been shown to inhibit bone resorption [128], induce collagen synthesis [129] and osteopontin and alkaline phosphatase production [130,131] in a variety of osteoblastic cell lines. The conflicting results may be due to differences in cell lines, particularly with regards to ET receptor expression. Although these *in vitro* data are in apparent conflict, the *in vivo* data support that ET-1 promotes bone formation [132]. Specifically, administration of an ET_A receptor antagonist in mice resulted in reduced bone mass [132].

ET-1 is secreted by normal prostate epithelial cells into the ejaculate [133–135] and is now considered a putative mediator of prostate carcinoma pathophysiology (as reviewed in [136]). The ectopic expression of ET-1 in the bone metastatic site by prostate carcinoma cells may enable ET-1 to influence the bone remodeling process locally. This is supported by the report that para-tibial injection of an amniotic cell line overexpressing ET-1 induced new bone formation in the tibiae of mice, which was diminished by blockade of ET_A receptor [137]. Additionally, administration of an ETA receptor antagonist diminished breast cancer-induced bone production in a murine model [138]. Furthermore, co-incubating the androgen-independent prostate carcinoma cell lines DU-145 and PC-3, but not the androgen-responsive cell line LNCaP, with bone slices induced ET-1 expression from the prostate carcinoma cells [103]. The DU-145 and PC-3 cell lines also induced osteoclastogenic activity that was blocked by anti-human ET-1 antibody. Taken together, these reports suggest that ET-1 may contribute to prostate carcinoma metastases-induced osteoblastic lesions. In apparent conflict with these models, is the observation that serum ET-1 levels are elevated in people with Paget's disease, which is characterized by low bone mineral density secondary to increased osteoclastic activity [139].

3.2.3. Bone morphogenetic proteins

BMPs are members of the transforming growth factor (TFG)-β superfamily. More than 30 BMPs have been identified to date [140]. While originally discovered because of their ability to induce new bone formation, BMPs are now recognized to perform many functions, particularly in the role of development, such as apoptosis, differentiation, proliferation and morphogenesis (as reviewed in [141–143]). BMPs are synthesized as large precursor molecules that undergo proteolytic cleavage to release the mature protein, which form active hetero- or homodimers [144,145]. BMPs bind to receptors (BMPR-IA and -IB) and a BMP type II receptor (BMPR-II), which induces Smad phosphorylation [146] resulting in modulation of gene regulation. Target genes of BMPs include osteoblast proteins such as OPG [147] and the osteoblast-specific transcription factor Cbfa-1 [148,149]. Several proteins that antagonize BMP action have been identified. For example, noggin and gremlin inhibit BMP-2, -4 and -7 by binding to them [150–152]. Furthermore, the BMPs themselves regulate their own inhibitors in an apparent negative feedback mechanism [153,154].

Many *in vitro* studies have demonstrated that BMPs induce osteogenic differentiation including the ability of BMP-7 (also called osteogenic protein-1; OP-1) to induce osteogenic differentiation of newborn rat calvarial cells and rat osteosarcoma cells [155–157]. The BMPs' osteogenic properties appear to be specific to the differentiation stage of the target cells. Specifically, BMPs can induced uncommitted stem cells [155,158,159] and myoblasts [160] to express osteoblast parameters such as alkaline phosphatase or osteocalcin expression [79,161]; whereas, BMPs do not stimulate mature osteoblasts or fibroblasts [158,162–164] to increase expression of these proteins. Examination of genetically modified mice provides further evidence of the importance of BMP in bone development. The *bmp7* homozygous null condition in mice is a postnatal lethal mutation and is associated with, in addition to renal and ocular abnormalities, retarded skeletal ossification [165]. In contrast, *bmp6* null mice are viable and fertile, and the skeletal elements of newborn and adult mutants are indistinguishable from wildtype [166]. However, careful examination of skeletogenesis in late gestation embryos reveals a consistent delay in ossification strictly confined to the developing sternum. Finally, mice with mutations of the *bmp5* gene have skeletal abnormalities and inefficient fracture repair [167]. Taken together, these data provide

evidence that BMPs are important regulators of the osteogenesis. Thus, dysregulation of their expression in the bone microenvironment would most likely impact bone remodeling.

A few studies have examined the expression of BMPs in normal and neoplastic prostate tissues. Using Northern analysis, Harris et al. [92] examined BMP-2, -3, -4 and -6 mRNA expression in human normal prostate and prostate carcinoma cell lines. They found that normal human prostate predominantly expressed BMP-4. The androgen-dependent non-metastatic LNCaP human prostate carcinoma cell line produced very low to undetectable levels of BMPs. Whereas, the aggressive androgen-independent PC-3 cell line expressed very high levels of BMP-3 and slightly lower levels of BMP-2, -4 and -6 compared to normal cells, but much higher than LNCaP cells. In support of these results, Weber et al. [168], using PCR analysis, identified 16 (73%) of 22 prostate carcinoma samples that were positive for BMP-7 mRNA compared to eight (57%) of 14 normal prostate tissue samples. In another PCR based analysis, Bentley et al. [169], found that several BMPs were expressed in both benign and malignant prostate tissue and in the PC3 and DU145 prostate carcinoma cell lines. BMP-6 expression was detected in the prostate tissue of over 50% of patients with clinically defined metastatic prostate adenocarcinoma, but was not detected in non-metastatic or benign prostate samples. In another study focused on BMP-6 mRNA and protein expression, Barnes et al. [170] observed that BMP-6 was produced by normal and neoplastic human prostate (radical prostatectomy specimens and human carcinoma cell lines DU145 and PC3). However, BMP-6 mRNA and protein expression was higher in prostate carcinoma as compared with adjacent normal prostate, with higher-grade tumors (Gleason score of 6 or more) having greater BMP-6 immunostaining than the lower-grade tumors (Gleason score of 4 or less). These results were consistent with a later study by Hamdy et al. [171], who reported that BMP-6 mRNA expression was detected exclusively in malignant epithelial cells in 20 of 21 patients (95%) with metastases, in 2 of 11 patients (18%) with localized cancer, and undetectable in 8 benign samples. In addition to BMP, there have been several reports that prostate carcinoma expresses BMP receptors. It appears that as prostate carcinoma progress, the cells down-regulate their own expression of BMP receptors [172,173], which may be a protective mechanism as it has been demonstrated that BMP-2 can inhibit prostate carcinoma cell proliferation [174]. Taken together, these observations demonstrate that prostate carcinoma cells produce increasing levels of BMPs as they progress to a more aggressive phenotype and suggest that the up-regulation of BMP expression in prostate carcinoma cells localized in the bone is a critical component of the mechanism of development of osteoblastic lesions at prostate carcinoma metastatic sites.

4. The effect of prostate carcinoma on the bone: Osteolytic

Although the osteoblastic component of prostate carcinoma metastases has received attention, limited research has been performed on the osteoclastic aspect of prostate carcinoma. Similar to the reports for breast cancer bone metastases [175,176], several lines of evidence suggest that resorption of bone is an important mediator of prostate carcinoma bone metastases. For example, administration of bisphosphonates, inhibitors of osteoclast activity, to patients with prostate carcinoma bone metastases relieves bone pain and lowers systemic indices of bone resorption [177–179]. Furthermore, administration of osteoclast inhibitors such as OPG or bisphosphonates prevents tumor establishment or diminished tumor burden in animal models [76,180–182]. It is not clear if bisphosphonates have a direct antitumor effect [183–185] or inhibit tumor growth through its ability to diminish osteoclast activity [186,187]. In some instances, it may be a combination of activities. As described above, in addition to serum levels of bone resorption markers being elevated in men with prostate carcinoma skeletal metastases, the lesions usually are demonstrated to have histological evidence of osteoclast activity. Thus, osteoclast activity may play an important role in development and progression of prostate carcinoma metastases. Prostate carcinoma cells secrete a variety of factors that may promote bone lysis, such as interleukin-6 (as reviewed in [188]) and PTHrP. However, it appears that these factors mediate their osteolytic effects through induction of a key pro-osteoclastogenic molecule, receptor activator of NFκB ligand (RANKL).

4.1. Receptor activator of NFkB ligand-OPG axis

A member of the tumor necrosis factor family, RANKL is initially expressed as a membrane anchored molecule; however, a small fraction of RANKL is released

through proteolytic cleavage from the cell surface as a soluble 245 amino acid homotrimeric molecule (sRANKL) [189]. Both soluble and membrane bound RANKL promote osteoclast formation and activation by binding to RANK on the osteoclast precursor membrane [189–193].

In addition to RANKL and RANK, another key modulator of osteoclastogenesis is osteoprotegerin (OPG)(also known as osteoclastogenesis inhibitory factor-OCIF) [102,194]. OPG serves as a decoy receptor that binds RANKL and thus blocks its ability to bind to RANK and induce osteoclastogenesis. In contrast to RANKL and RANK, whose expression is mainly restricted at low levels to the skeletal and immune systems, OPG is expressed in a variety of tissues, such as liver, lung, heart, kidney, stomach, intestines, skin and calvaria in mice and lung, heart, kidney and placenta in human [102,195–201]. In bone, OPG is mainly produced by osteoblastic lineage cells and its expression increases as the cells become more differentiated [199,202,203]. Administration of recombinant OPG to normal rodents resulted in increased bone mass [102,196] and completely prevented ovariectomy-induced bone loss without apparent adverse skeletal and extraskeletal side effects [102]. In fact, based on this activity, the balance ratio of RANKL to OPG appears to be very important in controlling the overall activity (i.e., lysis vs no lysis) that will be observed [204–206].

A number of reports have shown that osteoclastic bone resorptive lesions are important to the development of bone metastases in several cancer types including breast cancer, lung cancer and prostate carcinoma [207]. These cancers may induce osteoclast activity through secretion of IL-1α, PTHrP or PGE2 [208,209]. However, tumor-mediated osteolysis occurs indirectly through expression of molecules, such as PTHrP, that induce RANKL in osteoblasts [210,211]. This contrasts with the observations that giant cell tumors directly promote osteoclast activity via RANKL [212] and our observation that prostate carcinoma cells directly induce osteoclastogenesis through RANKL [76]. Another factor that may play a role in tumor-induced osteoclastogenesis is human macrophage inflammatory protein-1α (hMIP-1α), which has been shown to be produced by myeloma cells [213]. Because of the osteoclastic activity induced by many cancers, antiresorptive approaches such as administration of bisphosphonates or anti-PTHrP neutralizing antibody have been reported in breast cancer animal models to be able to block the tumor expansion in bone [214,215]. Furthermore, OPG has been recently shown to inhibit primary bone sarcoma-induced osteolysis and tumor-induced bone pain, but not tumor burden in mice [100]. However, OPG not only blocked osteolytic bone metastasis induced by human neuroblastoma NB-19 cells [216], but also reduced tumor burden in that model. In addition to OPG, a soluble form of RANK (sRANK) has been shown to inhibit myeloma-induced lytic lesions in murine models [217].

4.2. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are family of enzymes whose primary function is to degrade the extracellular matrix. MMPs contribute to metastatic invasion, including destruction of bone [218]. Prostate carcinomas and their cell lines express a large number of MMPs [219–226]. The initial functional data in prostate carcinoma bone metastasis that suggested bone remodeling is modulated through MMPs was provided by *in vitro* studies. Specifically, blocking MMP activity with 1,10-phenanthroline, a MMP inhibitor, diminished bone matrix degradation induced by PC-3 cells *in vitro* [227,228]. The importance of MMPs in bone metastasis has been further confirmed *in vivo*. An MMP inhibitor, batimistat, has been shown to inhibit development bone resorption *in vitro* and *in vivo* in murine models of breast [229] and prostate carcinoma [230]. The mechanism through which prostate carcinoma-produced MMPs induce bone resorption is not clear; however, it appears to involve induction of osteoclastogenesis as inhibition of MMPs reduced the number of osteoclasts associated with prostate tumor growth in human bone implants in mice [230].

5. Conclusions

A model summarizing the cross-talk between prostate carcinoma and the bone microenvironment that leads to development and progression of prostate carcinoma skeletal metastases is presented in Figure 1. The bone contributes many aspects of the metastatic cascade including chemotaxis, endothelial attachment, invasion and tumor proliferation. Once in the bone microenvironment, the prostate carcinoma cells modulate bone remodeling which favors tumor progression. The presence of many different active factors produced by both the bone and the prostate carcinoma cells that appear to contribute to the pathobiology of skeletal metastases

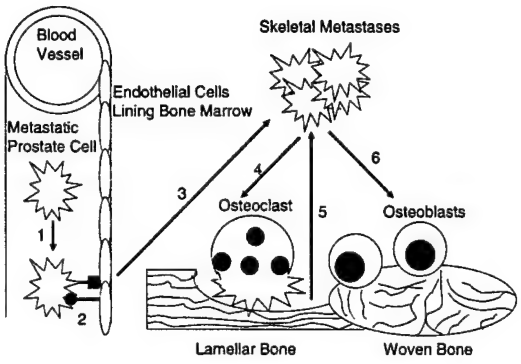

Figure 1. Model of cross-talk between prostate carcinoma cells and the bone microenvironment. The bone produces chemotactic factors that attract prostate carcinoma cells to migrate (1) through the vascular system towards the skeleton. The bone marrow endothelia displays adhesion molecules that complement those expressed by the prostate carcinoma cell, resulting in attachment of the cell (2). The prostate carcinoma cell extravasates and invades into the skeletal extracellular tissue (3), at which point it releases factors that stimulate osteoclastogenesis (4). The subsequent bone resorption is accompanied by release of growth factors that stimulate prostate carcinoma proliferation (5). The progressing prostate carcinoma releases factors that promote osteoblast production and inhibit osteoblast apoptosis (6) resulting in production of woven bone and the characteristic osteosclerotic lesion. This process continues in a cyclical fashion with continued induction of osteoclastic activity, carcinoma cell proliferation and bone production.

suggests that defining the mechanisms of prostate carcinoma skeletal metastases will be challenging. Continued research on how these interactions occur may lead to identification of targets to interrupt this cross-talk and prevent the establishment or progression of prostate cancer skeletal metastases.

Acknowledgements

This work was supported by USAMRMC Prostate carcinoma Research Program Grant # DAMD17-00-1-053, National Institutes of Health Grants SPORE 1 P50 CA69568 and T32 RR07008.

References

1. Landis SH, Murray T, Bolden S, Wingo PA: Cancer statistics, 1999. CA Cancer J Clin 49: 8–31, 1999
2. Abrams H, Spiro R, Goldstein N: Metastases in carcinoma. Cancer 3: 74–85, 1950
3. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, Mihatsch MJ: Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol 31: 578–583, 2000
4. Rana A, Chisholm GD, Khan M, Sekharjit SS, Merrick MV, Elton RA: Patterns of bone metastasis and their prognostic significance in patients with carcinoma of the prostate. Br J Urol 72: 933–936, 1993
5. Galasko CS: Skeletal metastases. Clin Orthop 1986: 18–30, 1986
6. Coleman RE: Skeletal complications of malignancy. Cancer 80: 1588–1594, 1997
7. Moul JW, Lipo DR: Prostate cancer in the late 1990s: Hormone refractory disease options. Urol Nurs 19: 125–131; quiz 132–123, 1999
8. Szostak MJ, Kyprianou N: Radiation-induced apoptosis: predictive and therapeutic significance in radiotherapy of prostate cancer (review). Oncol Rep 7: 699–706, 2000
9. Papapoulos SE, Hamdy NA, van der Pluijm G: Bisphosphonates in the management of prostate carcinoma metastatic to the skeleton. Cancer 88: 3047–3053, 2000
10. Paget S: The distribution of secondary growth in cancer of the breast. Lancet 1: 571–573, 1829
11. Tsingotjidou AS, Zotalis G, Jackson KR, Sawyers C, Puzas JE, Hicks DG, Reiter R, Lieberman JR: Development of an animal model for prostate cancer cell metastasis to adult human bone. Anticancer Res 21: 971–978, 2001
12. Magro C, Orr FW, Manishen WJ, Sivananthan K, Mokashi SS: Adhesion, chemotaxis, and aggregation of Walker carcinosarcoma cells in response to products of resorbing bone. J Natl Cancer Inst 74: 829–838, 1985
13. Orr W, Varani J, Gondex MK, Ward PA, Mundy GR: Chemotactic responses of tumor cells to products of resorbing bone. Science 203: 176–179, 1979
14. Orr FW, Varani J, Gondek MD, Ward PA, Mundy GR: Partial characterization of a bone-derived chemotactic factor for tumor cells. Am J Pathol 99: 43–52, 1980
15. Wass JA, Varani J, Piontek GE, Ward PA, Orr FW: Responses of normal and malignant cells to collagen, collagen-derived peptides and the C5-related tumor cell chemotactic peptide. Cell Differ 10: 329–332, 1981
16. Jacob K, Webber M, Benayahu D, Kleinman HK: Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Res 59: 4453–4457, 1999
17. Hullinger TG, McCauley LK, DeJoode ML, Somerman MJ: Effect of bone proteins on human prostate cancer cell lines *in vitro*. Prostate 36: 14–22, 1998
18. Rajan R, Vanderslice R, Kapur S, Lynch J, Thompson R, Djakiew D: Epidermal growth factor (EGF) promotes chemomigration of a human prostate tumor cell line, and EGF immunoreactive proteins are present at sites of metastasis in the stroma of lymph nodes and medullary bone. Prostate 28: 1–9, 1996
19. Somlyo AV, Bradshaw D, Ramos S, Murphy C, Myers CE, Somlyo AP: Rho-kinase inhibitor retards migration and *in vivo* dissemination of human prostate cancer cells. Biochem Biophys Res Commun 269: 652–659, 2000
20. Baggiolini M: Chemokines and leukocyte traffic. Nature 392: 565–568, 1998

21. Rossi D, Zlotnik A: The biology of chemokines and their receptors. Annu Rev Immunol 18: 217–242, 2000
22. Strieter RM: Chemokines: Not just leukocyte chemoattractants in the promotion of cancer. Nat Immunol 2: 285–286, 2001
23. Aiuti A, Tavian M, Cipponi A, Ficara F, Zappone E, Hoxie J, Peault B, Bordignon C: Expression of CXCR4, the receptor for stromal cell-derived factor-1 on fetal and adult human lympho-hematopoietic progenitors. Eur J Immunol 29: 1823–1831, 1999
24. Kim CH, Broxmeyer HE: SLC/exodus2/6Ckine/TCA4 induces chemotaxis of hematopoietic progenitor cells: Differential activity of ligands of CCR7, CXCR3, or CXCR4 in chemotaxis vs. suppression of progenitor proliferation. J Leukoc Biol 66: 455–461, 1999
25. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T: Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382: 635–638, 1996
26. Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, Lider O, Alon R, Zipori D, Lapidot T: Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283: 845–848, 1999
27. Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, Arenzana-Seisdedos F, Magerus A, Caruz A, Fujii N, Nagler A, Lahav M, Szyper-Kravitz M, Zipori D, Lapidot T: Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J Clin Invest 106: 1331–1339, 2000
28. Imai K, Kobayashi M, Wang J, Shinobu N, Yoshida H, Hamada J, Shindo M, Higashino F, Tanaka J, Asaka M, Hosokawa M: Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. Br J Haematol 106: 905–911, 1999
29. Moore BB, Arenberg DA, Stoy K, Morgan T, Addison CL, Morris SB, Glass M, Wilke C, Xue YY, Sitterding S, Kunkel SL, Burdick MD, Strieter RM: Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. Am J Pathol 154: 1503–1512, 1999
30. Reiland J, Furcht LT, McCarthy JB: CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. Prostate 41: 78–88, 1999
31. Gupta SK, Pillarisetti K: Cutting edge: CXCR4-Lo: Molecular cloning and functional expression of a novel human CXCR4 splice variant. J Immunol 163: 2368–2372, 1999
32. Sehgal A, Ricks S, Boynton AL, Warrick J, Murphy GP: Molecular characterization of CXCR-4: A potential brain tumor-associated gene. J Surg Oncol 69: 239–248, 1998
33. Mohle R, Failenschmid C, Bautz F, Kanz L: Overexpression of the chemokine receptor CXCR4 in B cell chronic lymphocytic leukemia is associated with increased functional response to stromal cell-derived factor-1 (SDF-1). Leukemia 13: 1954–1959, 1999
34. Taichman R, McCauley L, Taichman N: Use of the SDF-1/CXCR4 pathway in prostate cancer metastasis to bone. Blood 96: 571a, 2000
35. Muller C, Homey B, Sato H, Ge N, Catron D, Buchanan M, McClanahan T, Murphy E, Yuan W, Wagners S, Barrera J, Mohar A, Verastegui E, Zlotnik A: Involvement of chemokine receptors in breast cancer metastasis. Nature 410: 50–56, 2001
36. Liotta LA: An attractive force in metastasis. Nature 410: 24–25, 2001
37. Miyasaka M: Cancer metastasis and adhesion molecules. Clin Orthop 312: 10–18, 1995
38. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM: Interactions between cancer cells and the endothelium in metastasis. J Pathol 190: 310–329, 2000
39. Pauli BU, Augustin-Voss HG, El-Sabban ME, Johnson RC, Hammer DA: Organ-preference of metastasis. Cancer and Metastasis Rev 9: 175–189, 1990
40. Deroock IB, Pennington ME, Sroka TC, Lam KS, Bowden GT, Bair EL, Cress AE: Synthetic peptides inhibit adhesion of human tumor cells to extracellular matrix proteins. Cancer Res 61: 3308–3313, 2001
41. vanderPluijm G, Vloedgraven H, Papapoulos S, Lowik C, Grzesik W, Kerr J, Robey PG: Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. Lab Inves 77: 665–675, 1997
42. Lehr JE, Pienta KJ: Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line (see comments). J Natl Cancer Inst 90: 118–123, 1998
43. Cooper CR, Pienta KJ: Cell adhesion and chemotaxis in prostate cancer metastasis to bone: a minireview. Prostate Canc Prostatic Dis 3: 6–12, 2000
44. Cooper CR, Mclean L, Walsh M, Taylor J, Hayasaka S, Bhatia J, Pienta KJ: Preferential adhesion to prostate cancer cells to bone is mediated by binding to bone marrow endothelial cells as compared to extracellular matrix components *in vitro*. Clin Cancer Res 6: 4839–4847, 2000
45. Scott LJ, Clarke NW, George NJ, Shanks JH, Testa NG, Lang SH: Interactions of human prostatic epithelial cells with bone marrow endothelium: binding and invasion. Br J Cancer 84: 1417–1423, 2001
46. Pienta KJ, Naik H, Akhtar A, Yamazaki K, Replogle TS, Lehr J, Donat TL, Tait L, Hogan V, Raz A: Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. J Natl Cancer Inst 87: 348–353, 1995
47. Romanov VI, Goligorsky MS: RGD-recognizing integrins mediate interactions of human prostate carcinoma cells with endothelial cells *in vitro*. Prostate 39: 108–118, 1999
48. Cooper CR, McLean L, Mucci NR, Poncza P, Pienta KJ: Prostate cancer cell adhesion to quiescent endothelial cells is not mediated by beta-1 integrin subunit. Anticancer Res 20: 4159–4162, 2000
49. Simpson MA, Reiland J, Burger SR, Furch LT, Spice AP, Theodore R. Oegema J, McCarthy JB: Hyaluronan synthase elevation in metastatic prostate carcinoma cells correlates with hyaluronan surface retention, a prerequisite for

rapid adhesion to bone marrow endothelial cells. J Biol Chem 276: 17949–17957, 2001
50. Kierszenbaum AL, Rivkin E, Chang PL, Tres LL, Olsson CA: Galactosyl receptor, a cell surface C-type lectin of normal and tumoral prostate epithelial cells with binding affinity to endothelial cells. Prostate 43: 175–183, 2000
51. Kostenuik PJ, Sanchez-Sweatman O, Orr FW, Singh G: Bone cell matrix promotes the adhesion of human prostatic carcinoma cells via the alpha 2 beta 1 integrin. Clin Exp Metastasis 14: 19–26, 1996
52. Festuccia C, Bologna M, Gravina GL, Guerra F, Angelucci A, Villonava I, Millimaggi D, Teti A: Osteoblast conditioned media contained TGF-beta1 and modulat the migration of prostate tumor cells and their interactions with extracellular matrix components. Int J Cancer 81: 395–403, 1999
53. Kostenuik PJ, Singh G, Orr FW: Transforming growth factor-beta upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. Clin Exp Metastasis 15: 41–52, 1997
54. Martinez J, Fuentes M, Cambiazo V, Santibanez JF: Bone extracellular matrix stimulates invasiveness of estrogen-responsive human mammary MCF-7 cells. Int J Cancer 83: 278–282, 1999
55. Linkhart TA, Mohan S, Baylink DJ: Growth factors for bone growth and repair: IGF, TGF beta and BMP. Bone 19: 1S–12S, 1996
56. Manishen WJ, Sivananthan K, Orr FW: Resorbing bone stimulates tumor cell growth. A role for the host microenvironment in bone metastasis. Am J Pathol 123: 39–45, 1986
57. Ritchie CK, Andrews LR, Thomas KG, Tindall DJ, Fitzpatrick LA: The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: Implications for the development of metastatic disease. Endocrinology 138: 1145–1150, 1997
58. Song Z, Powell WC, Kasahara N, van Bokhoven A, Miller GJ, Roy-Burman P: The effect of fibroblast growth factor 8, isoform b, on the biology of prostate carcinoma cells and their interaction with stromal cells. Cancer Res 60: 6730–6736, 2000
59. Desruisseau S, Ghazarossian-Ragni E, Chinot O, Martin PM: Divergent effect of TGFbeta1 on growth and proteolytic modulation of human prostatic-cancer cell lines. Int J Cancer 66: 796–801, 1996
60. Djavan B, Waldert M, Seitz C, Marberger M: Insulin-like growth factors and prostate cancer. World J Urol 19: 225–233, 2001
61. Peehl DM, Cohen P, Rosenfeld RG: The insulin-like growth factor system in the prostate. World J Urol 13: 306–311, 1995
62. Cohen P, Peehl DM, Lamson G, Rosenfeld RG: Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins in primary cultures of prostate epithelial cells. J Clin Endocrinol Metab 73: 401–407, 1991
63. Festuccia C, Giunciuglio D, Guerra F, Villanova I, Angelucci A, Manduca P, Teti A, Albini A, Bologna M: Osteoblasts modulate secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in human prostate cancer cells promoting migration and matrigel invasion. Oncol Res 11: 17–31, 1999
64. Lang SH, Clarke NW, George NJ, Allen TD, Testa NG: Interaction of prostate epithelial cells from benign and malignant tumor tissue with bone-marrow stroma. Prostate 34: 203–213, 1998
65. Gleave ME, Hsieh JT, von Eschenbach AC, Chung LW: Prostate and bone fibroblasts induce human prostate cancer growth *in vivo*: Implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis. J Urol 147: 1151–1159, 1992
66. Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW: Acceleration of human prostate cancer growth *in vivo* by factors produced by prostate and bone fibroblasts. Cancer Res 51: 3753–3761, 1991
67. Yamashita K, Aoki Y, Hiroshima K: Metastatic epidural bony tumor causing spinal cord compression: A case report. Clin Orthop 1996: 231–235, 1996
68. Munk PL, Poon PY, O'Connell JX, Janzen D, Coupland D, Kwong JS, Gelmon K, Worsley D: Osteoblastic metastases from breast carcinoma with false-negative bone scan. Skeletal Radiol 26: 434–437, 1997
69. Berruti A, Piovesan A, Torta M, Raucci CA, Gorzegno G, Paccotti P, Dogliotti L, Angeli A: Biochemical evaluation of bone turnover in cancer patients with bone metastases: Relationship with radiograph appearances and disease extension. Br J Cancer 73: 1581–1587, 1996
70. Vinholes J, Coleman R, Eastell R: Effects of bone metastases on bone metabolism: Implications for diagnosis, imaging and assessment of response to cancer treatment. Cancer Treat Rev 22: 289–331, 1996
71. Urwin GH, Percival RC, Harris S, Beneton MN, Williams JL, Kanis JA: Generalised increase in bone resorption in carcinoma of the prostate. Br J Urol 57: 721–723, 1985
72. Roudier M, Sherrard D, True L, Ott-Ralp S, Meligro C, MBerrie M, Soo C, Felise D, Quinn JE, Vessella R: Heterogenous bone histomorphometric patterns in metastatic prostate cancer. J Bone Miner Res 15S1: S567, 2000
73. Clarke NW, McClure J, George NJ: Osteoblast function and osteomalacia in metastatic prostate cancer. Eur Urol 24: 286–290, 1993
74. Charhon SA, Chapuy MC, Delvin EE, Valentin-Opran A, Edouard CM, Meunier PJ: Histomorphometric analysis of sclerotic bone metastases from prostatic carcinoma special reference to osteomalacia. Cancer 51: 918–924, 1983
75. Carlin BI, Andriole GL: The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. Cancer 88: 2989–2994, 2000
76. Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J, Keller ET: Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 107: 1235–1244, 2001
77. Maeda H, Koizumi M, Yoshimura K, Yamauchi T, Kawai T, Ogata E: Correlation between bone metabolic markers and bone scan in prostatic cancer. J Urol 157: 539–543, 1997

78. Demers LM, Costa L, Lipton A: Biochemical markers and skeletal metastases. Cancer 88: 2919–2926, 2000
79. Karsenty G: Bone formation and factors affecting this process. Matrix Biol 19: 85–89, 2000
80. Parfitt AM: The mechanism of coupling: A role for the vasculature. Bone 26: 319–323, 2000
81. Boyce BF, Hughes DE, Wright KR, Xing L, Dai A: Recent advances in bone biology provide insight into the pathogenesis of bone diseases. Lab Invest 79: 83–94, 1999
82. Rosol TJ: Pathogenesis of bone metastases: Role of tumor-related proteins. J Bone Miner Res 15: 844–850, 2000
83. Blomme EA, Dougherty KM, Pienta KJ, Capen CC, Rosol TJ, McCauley LK: Skeletal metastasis of prostate adenocarcinoma in rats: Morphometric analysis and role of parathyroid hormone-related protein. Prostate 39: 187–197, 1999
84. Yang J, Fizazi K, Peleg S, Sikes CR, Raymond AK, Jamal N, Hu M, Olive M, Martinez LA, Wood CG, Logothetis CJ, Karsenty G, Navone NM: Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. Cancer Res 61: 5652–5659, 2001
85. Lin DL, Tarnowski CP, Zhang J, Dai J, Rohn E, Patel AH, Morris MD, Keller ET: Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes *in vitro*. Prostate 47: 212–221, 2001
86. Boyce BF, Yoneda T, Guise TA: Factors regulating the growth of metastatic cancer in bone. Endocr Relat Cancer 6: 333–347, 1999
87. Deftos LJ: Prostate carcinoma: Production of bioactive factors. Cancer 88: 3002–3008, 2000
88. Yoneda T: Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur J Cancer 34: 240–245, 1998
89. Goltzman D, Bolivar I, Rabbani SA: Studies on the pathogenesis of osteoblastic metastases by prostate cancer. Adv Exp Med Biol 324: 165–171, 1992
90. Koutsilieris M, Rabbani SA, Goltzman D: Selective osteoblast mitogens can be extracted from prostatic tissue. Prostate 9: 109–115, 1986
91. Autzen P, Robson CN, Bjartell A, Malcolm AJ, Johnson MI, Neal DE, Hamdy FC: Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br J Cancer 78: 1219–1223, 1998
92. Harris SE, Harris MA, Mahy P, Wozney J, Feng JQ, Mundy GR: Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate 24: 204–211, 1994
93. Hullinger TG, Taichman RS, Linseman DA, Somerman MJ: Secretory products from PC-3 and MCF-7 tumor cell lines upregulate osteopontin in MC3T3-E1 cells. J Cell Biochem 78: 607–616, 2000
94. Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW: Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat Med 1: 944–949, 1995
95. Kimura G, Sugisaki Y, Masugi Y, Nakazawa N: Calcification in human osteoblasts cultured in medium conditioned by the prostatic cancer cell line PC-3 and prostatic acid phosphatase. Urol Int 48: 25–30, 1992
96. Karaplis AC, Vautour L: Parathyroid hormone-related peptide and the parathyroid hormone/parathyroid hormone-related peptide receptor in skeletal development. Curr Opin Nephrol Hypertens 6: 308–313, 1997
97. Cornish J, Callon KE, Lin C, Xiao C, Moseley JM, Reid IR: Stimulation of osteoblast proliferation by C-terminal fragments of parathyroid hormone-related protein. J Bone Miner Res 14: 915–922, 1999
98. Rabbani SA, Gladu J, Mazar AP, Henkin J, Goltzman D: Induction in human osteoblastic cells (SaOS2) of the early response genes fos, jun, and myc by the amino terminal fragment (ATF) of urokinase. J Cell Physiol 172: 137–145, 1997
99. Killian CS, Corral DA, Kawinski E, Constantine RI: Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. Biochem Biophys Res Commun 192: 940–947, 1993
100. Honore P, Luger NM, Sabino MA, Schwei MJ, Rogers SD, Mach DB, O'Keefe PF, Ramnaraine ML, Clohisy DR, Mantyh PW: Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat Med 6: 521–528, 2000
101. Guise TA: Molecular mechanisms of osteolytic bone metastases. Cancer 88: 2892–2898, 2000
102. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ: Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89: 309–319, 1997
103. Chiao JW, Moonga BS, Yang YM, Kancherla R, Mittelman A, Wu-Wong JR, Ahmed T: Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption. Br J Cancer 83: 360–365, 2000
104. Laitinen M, Marttinen A, Aho AJ, Lindholm TS: Bone morphogenetic protein in bone neoplasms: Comparison of different detection methods. Eur Surg Res 30: 168–174, 1998
105. Raval P, Hsu HH, Schneider DJ, Sarras MP Jr., Masuhara K, Bonewald LF, Anderson HC: Expression of bone morphogenetic proteins by osteoinductive and non-osteoinductive human osteosarcoma cells. J Dent Res 75: 1518–1523, 1996
106. Wlosarski K, Reddi AH: Tumor cells stimulate *in vivo* periosteal bone formation. Bone Miner 2: 185–192, 1987
107. Strewler GJ: The physiology of parathyroid hormone-related protein. N Engl J Med 342: 177–185, 2000
108. Lanske B, Amling M, Neff L, Guiducci J, Baron R, Kronenberg HM: Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. J Clin Invest 104: 399–407, 1999
109. Amizuka N, Henderson JE, White JH, Karaplis AC, Goltzman D, Sasaki T, Ozawa H: Recent studies on the biological action of parathyroid hormone (PTH)-related

peptide (PTHrP) and PTH/PTHrP receptor in cartilage and bone. Histol Histopathol 15: 957–970, 2000
110. Iwamura M, Abrahamsson PA, Schoen S, Cockett AT, Deftos LJ: Immunoreactive parathyroid hormone-related protein is present in human seminal plasma and is of prostate origin. J Androl 15: 410–414, 1994
111. Iwamura M, di Sant'Agnese PA, Wu G, Benning CM, Cockett AT, Deftos LJ, Abrahamsson PA: Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. Cancer Res 53: 1724–1726, 1993
112. Asadi F, Farraj M, Sharifi R, Malakouti S, Antar S, Kukreja S: Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia. Hum Pathol 27: 1319–1323, 1996
113. Iwamura M, Gershagen S, Lapets O, Moynes R, Abrahamsson PA, Cockett AT, Deftos LJ, di Sant'Agnese PA: Immunohistochemical localization of parathyroid hormone-related protein in prostatic intraepithelial neoplasia. Hum Pathol 26: 797–801, 1995
114. Dougherty KM, Blomme EA, Koh AJ, Henderson JE, Pienta KJ, Rosol TJ, McCauley LK: Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. Cancer Res 59: 6015–6022, 1999
115. Burton PB, Moniz C, Knight DE: Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma. Biochem Biophys Res Commun 167: 1134–1138, 1990
116. Bouizar Z, Spyratos F, De vernejoul MC: The parathyroid hormone-related protein (PTHrP) gene: use of downstream TATA promotor and PTHrP 1-139 coding pathways in primary breast cancers vary with the occurrence of bone metastasis. J Bone Miner Res 14: 406–414, 1999
117. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA: TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest 103: 197–206, 1999
118. Iwamura M, Abrahamsson PA, Foss KA, Wu G, Cockett AT, Deftos LJ: Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. Urology 43: 675–679, 1994
119. Henderson JE, Amizuka N, Warshawsky H, Biasotto D, Lanske BM, Goltzman D, Karaplis AC: Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Mol Cell Biol 15: 4064–4075, 1995
120. Aarts MM, Levy D, He B, Stregger S, Chen T, Richard S, Henderson JE: Parathyroid hormone-related protein interacts with RNA. J Biol Chem 274: 4832–4838, 1999
121. Ye Y, Falzon M, Seitz PK, Cooper CW: Overexpression of parathyroid hormone-related protein promotes cell growth in the rat intestinal cell line IEC-6. Regul Pept 99: 169–174, 2001
122. Massfelder T, Dann P, Wu TL, Vasavada R, Helwig JJ, Stewart AF: Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. Proc Natl Acad Sci USA 94: 13630–13635, 1997
123. Cramer SD, Chen Z, Peehl DM: Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: Inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. J Urol 156: 526–531, 1996
124. Ye Y, Seitz PK, Cooper CW: Parathyroid hormone-related protein overexpression in the human colon cancer cell line HT-29 enhances adhesion of the cells to collagen type I. Regul Pept 101: 19–23, 2001
125. Stjernquist M: Endothelins–vasoactive peptides and growth factors. Cell Tissue Res 292: 1–9, 1998
126. Takuwa Y, Ohue Y, Takuwa N, Yamashita K: Endothelin-1 activates phospholipase C and mobilizes Ca^{2+} from extra- and intracellular pools in osteoblastic cells. Am J Physiol 257: E797–803, 1989
127. Hiruma Y, Inoue A, Shiohama A, Otsuka E, Hirose S, Yamaguchi A, Hagiwara H: Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am J Physiol 275: R1099–1105, 1998
128. Zaidi M, Alam AS, Bax BE, Shankar VS, Bax CM, Gill JS, Pazianas M, Huang CL, Sahinoglu T, Moonga BS, et al.: Role of the endothelial cell in osteoclast control: New perspectives. Bone 14: 97–102, 1993
129. Tatrai A, Foster S, Lakatos P, Shankar G, Stern PH: Endothelin-1 actions on resorption, collagen and noncollagen protein synthesis, and phosphatidylinositol turnover in bone organ cultures. Endocrinology 131: 603–607, 1992
130. Shioide M, Noda M: Endothelin modulates osteopontin and osteocalcin messenger ribonucleic acid expression in rat osteoblastic osteosarcoma cells. J Cell Biochem 53: 176–180, 1993
131. Kasperk CH, Borcsok I, Schairer HU, Schneider U, Nawroth PP, Niethard FU, Ziegler R: Endothelin-1 is a potent regulator of human bone cell metabolism *in vitro*. Calcif Tissue Int 60: 368–374, 1997
132. Tsukahara H, Hori C, Hiraoka M, Yamamoto K, Ishii Y, Mayumi M: Endothelin subtype A receptor antagonist induces osteopenia in growing rats. Metabolism 47: 1403–1407, 1998
133. Langenstroer P, Tang R, Shapiro E, Divish B, Opgenorth T, Lepor H: Endothelin-1 in the human prostate: Tissue levels, source of production and isometric tension studies. J Urol 150: 495–499, 1993
134. Walden PD, Ittmann M, Monaco ME, Lepor H: Endothelin-1 production and agonist activities in cultured prostate-derived cells: Implications for regulation of endothelin bioactivity and bioavailability in prostatic hyperplasia. Prostate 34: 241–250, 1998
135. Casey ML, Byrd W, MacDonald PC: Massive amounts of immunoreactive endothelin in human seminal fluid. J Clin Endocrinol Metab 74: 223–225, 1992
136. Nelson JB, Carducci MA: The role of endothelin-1 and endothelin receptor antagonists in prostate cancer. BJU Int 85(Suppl 2): 45–48, 2000
137. Nelson JB, Nguyen SH, Wu-Wong JR, Opgenorth TJ, Dixon DB, Chung LW, Inoue N: New bone formation in an osteoblastic tumor model is increased by endothelin-1

overexpression and decreased by endothelin A receptor blockade. Urology 53: 1063–1069, 1999
138. Yin J, Grubbs B, Cui Y, Weu-Wong J, Wessale J, Padley R, Guise T: Endothelin A receptor blockade inhibits osteoblastic metastases. J Bone Miner Res 15: S201, 2000
139. Tarquini R, Perfetto F, Tarquini B: Endothelin-1 and Paget's bone disease: Is there a link? Calcif Tissue Int 63: 118–120, 1998
140. Ducy P, Karsenty G: The family of bone morphogenetic proteins. Kidney Int 57: 2207–2214, 2000
141. Reddi AH: Bone morphogenetic proteins: An unconventional approach to isolation of first mammalian morphogens. Cytokine Growth Factor Rev 8: 11–20, 1997
142. Hogan BL: Bone morphogenetic proteins in development. Curr Opin Genet Dev 6: 432–438, 1996
143. Hall BK, Miyake T: All for one and one for all: condensations and the initiation of skeletal development. Bioessays 22: 138–147, 2000
144. Wozney JM: The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 32: 160–167, 1992
145. Suzuki A, Kaneko E, Maeda J, Ueno N: Mesoderm induction by BMP-4 and -7 heterodimers. Biochem Biophys Res Commun 232: 153–156, 1997
146. Wrana JL: Regulation of Smad activity. Cell 100: 189–192, 2000
147. Wan M, Shi X, Feng X, Cao X: Transcriptional mechanisms of bone morphogenetic protein induced osteoprotegrin gene expression. J Biol Chem 276: 10119–10125, 2001
148. Tsuji K, Ito Y, Noda M: Expression of the PEBP2alphaA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone 22: 87–92, 1998
149. Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL: Differentiation of human marrow stromal precursor cells: Bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res 14: 1522–1535, 1999
150. Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, Stahl N, Jilka RL, Manolagas SC: Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J Bone Miner Res 15: 663–673, 2000
151. Zimmerman LB, De Jesus-Escobar JM, Harland RM: The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86: 599–606, 1996
152. Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM: The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development 126: 5515–5522, 1999
153. Nifuji A, Noda M: Coordinated expression of noggin and bone morphogenetic proteins (BMPs) during early skeletogenesis and induction of noggin expression by BMP-7. J Bone Miner Res 14: 2057–2066, 1999
154. Nifuji A, Kellermann O, Noda M: Noggin expression in a mesodermal pluripotent cell line C1 and its regulation by BMP. J Cell Biochem 73: 437–444, 1999
155. Li IW, Cheifetz S, McCulloch CA, Sampath KT, Sodek J: Effects of osteogenic protein-1 (OP-1, BMP-7) on bone matrix protein expression by fetal rat calvarial cells are differentiation stage specific. J Cell Physiol 169: 115–125, 1996
156. Asahina I, Sampath TK, Nishimura I, Hauschka PV: Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123: 921–933, 1993
157. Maliakal JC, Asahina I, Hauschka PV, Sampath TK: Osteogenic protein-1 (BMP-7) inhibits cell proliferation and stimulates the expression of markers characteristic of osteoblast phenotype in rat osteosarcoma (17/2.8) cells. Growth Factors 11: 227–234, 1994
158. Yamaguchi A, Ishizuya T, Kintou N, Wada Y, Katagiri T, Wozney JM, Rosen V, Yoshiki S: Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 220: 366–371, 1996
159. Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T: The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172: 295–299, 1990
160. Katagiri T, Akiyama S, Namiki M, Komaki M, Yamaguchi A, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T: Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. Exp Cell Res 230: 342–351, 1997
161. Ducy P, Schinke T, Karsenty G: The osteoblast: A sophisticated fibroblast under central surveillance. Science 289: 1501–1504, 2000
162. Knutsen R, Wergedal JE, Sampath TK, Baylink DJ, Mohan S: Osteogenic protein-1 stimulates proliferation and differentiation of human bone cells *in vitro*. Biochem Biophys Res Commun 194: 1352–1358, 1993
163. Kim KJ, Itoh T, Kotake S: Effects of recombinant human bone morphogenetic protein-2 on human bone marrow cells cultured with various biomaterials. J Biomed Mater Res 35: 279–285, 1997
164. Groeneveld EH, Burger EH: Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol 142: 9–21, 2000
165. Jena N, Martin-Seisdedos C, McCue P, Croce CM: BMP7 null mutation in mice: Developmental defects in skeleton, kidney, and eye. Exp Cell Res 230: 28–37, 1997
166. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ: Mice lacking Bmp6 function. Dev Genet 22: 321–339, 1998
167. Kingsley DM, Bland AE, Grubber JM, Marker PC, Russell LB, Copeland NG, Jenkins NA: The mouse short ear skeletal morphogenesis locus is associated with defects

in a bone morphogenetic member of the TGF beta superfamily. Cell 71: 399–410, 1992

168. Weber KL, Bolander ME, Rock MG, Pritchard D, Sarkar G: Evidence for the upregulation of osteogenic protein-1 mRNA expression in musculoskeletal neoplasms. J Orthop Res 16: 8–14, 1998
169. Bentley H, Hamdy FC, Hart KA, Seid JM, Williams JL, Johnstone D, Russell RG: Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. Br J Cancer 66: 1159–1163, 1992
170. Barnes J, Anthony CT, Wall N, Steiner MS: Bone morphogenetic protein-6 expression in normal and malignant prostate. World J Urol 13: 337–343, 1995
171. Hamdy FC, Autzen P, Robinson MC, Horne CH, Neal DE, Robson CN: Immunolocalization and messenger RNA expression of bone morphogenetic protein-6 in human benign and malignant prostatic tissue. Cancer Res 57: 4427–4431, 1997
172. Kim IY, Lee DH, Ahn HJ, Tokunaga H, Song W, Devereaux LM, Jin D, Sampath TK, Morton RA: Expression of bone morphogenetic protein receptors type-IA, -IB and -II correlates with tumor grade in human prostate cancer tissues. Cancer Res 60: 2840–2844, 2000
173. Ide H, Katoh M, Sasaki H, Yoshida T, Aoki K, Nawa Y, Osada Y, Sugimura T, Terada M: Cloning of human bone morphogenetic protein type IB receptor (BMPR-IB) and its expression in prostate cancer in comparison with other BMPRs (published erratum appears in Oncogene 1997 Aug 28;15(9):1121). Oncogene 14: 1377–1382, 1997
174. Ide H, Yoshida T, Matsumoto N, Aoki K, Osada Y, Sugimura T, Terada M: Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. Cancer Res 57: 5022–5027, 1997
175. Mundy GR, Yoneda T, Hiraga T: Preclinical studies with zoledronic acid and other bisphosphonates: Impact on the bone microenvironment. Semin Oncol 28: 35–44, 2001
176. Theriault RL, Hortobagyi GN: The evolving role of bisphosphonates. Semin Oncol 28: 284–290, 2001
177. Pelger RC, Hamdy NA, Zwinderman AH, Lycklama a Nijeholt AA, Papapoulos SE: Effects of the bisphosphonate olpadronate in patients with carcinoma of the prostate metastatic to the skeleton. Bone 22: 403–408, 1998
178. Garnero P, Buchs N, Zekri J, Rizzoli R, Coleman RE, Delmas PD: Markers of bone turnover for the management of patients with bone metastases from prostate cancer. Br J Cancer 82: 858–864, 2000
179. Heidenreich A, Hofmann R, Engelmann UH: The use of bisphosphonate for the palliative treatment of painful bone metastasis due to hormone refractory prostate cancer (In Process Citation). J Urol 165: 136–140, 2001
180. Stearns ME, Wang M: Effects of alendronate and taxol on PC-3 ML cell bone metastases in SCID mice. Inv Met 16: 116–131, 1996
181. Sun YC, Geldof AA, Newling DW, Rao BR: Progression delay of prostate tumor skeletal metastasis effects by bisphosphonates. J Urol 148: 1270–1273, 1992
182. Wang M, Stearns ME: Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice. Differentiation 48: 115–125, 1991
183. Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, Delmas P, Delaisse JM, Clezardin P: Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res 60: 2949–2954, 2000
184. Boissier S, Magnetto S, Frappart L, Cuzin B, Ebetino FH, Delmas PD, Clezardin P: Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Res 57: 3890–3894, 1997
185. Lee MV, Fong EM, Singer FR, Guenette RS: Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res 61: 2602–2608, 2001
186. Diel IJ: Antitumour effects of bisphosphonates: first evidence and possible mechanisms. Drugs 59: 391–399, 2000
187. Hiraga T, Williams PJ, Mundy GR, Yoneda T: The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. Cancer Res 61: 4418–4424, 2001
188. Smith PC, Hobish A, Lin D, Culig Z, Keller ET: Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev 12: 33–40, 2001
189. Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP: Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)- converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. J Biol Chem 274: 13613–13618, 1999
190. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93: 165–176, 1998
191. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM: OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397: 315–323, 1999
192. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T: Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95: 3597–3602, 1998
193. Fuller K, Wong B, Fox S, Choi Y, Chambers TJ: TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J Exp Med 188: 997–1001, 1998

194. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, Higashio K: Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 234: 137–142, 1997
195. Tan KB, Harrop J, Reddy M, Young P, Terrett J, Emery J, Moore G, Truneh A: Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. Gene 204: 35–46, 1997
196. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K: Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. Endocrinology 139: 1329–1337, 1998
197. Kwon BS, Wang S, Udagawa N, Haridas V, Lee ZH, Kim KK, Oh KO, Greene J, Li Y, Su J, Gentz R, Aggarwal BB, Ni J: TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption. FASEB J 12: 845–854, 1998
198. Yun TJ, Chaudhary PM, Shu GL, Frazer JK, Ewings MK, Schwartz SM, Pascual V, Hood LE, Clark EA: OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. J Immunol 161: 6113–6121, 1998
199. Hofbauer LC, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S: Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. Biochem Biophys Res Commun 250: 776–781, 1998
200. Hofbauer LC, Heufelder AE: Osteoprotegerin and its cognate ligand: a new paradigm of osteoclastogenesis. Eur J Endocrinol 139: 152–154, 1998
201. Vidal NO, Brandstrom H, Jonsson KB, Ohlsson C: Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. J Endocrinol 159: 191–195, 1998
202. Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn JM, Niforas P, Ng KW, Martin TJ, Gillespie MT: Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. Bone 25: 525–534, 1999
203. Nagai M, Sato N: Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation. Biochem Biophys Res Commun 257: 719–723, 1999
204. Thomas GP, Baker SU, Eisman JA, Gardiner EM: Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. J Endocrinol 170: 451–460, 2001
205. Hofbauer LC, Heufelder AE, Erben RG: Osteoprotegerin, RANK, and RANK ligand: the good, the bad, and the ugly in rheumatoid arthritis. J Rheumatol 28: 685–687, 2001
206. Fazzalari NL, Kuliwaba JS, Atkins GJ, Forwood MR, Findlay DM: The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. J Bone Miner Res 16: 1015–1027, 2001
207. Yoneda T, Sasaki A, Mundy GR: Osteolytic bone metastasis in breast cancer. Breast Cancer Res Treat 32: 73–84, 1994
208. Akatsu T, Ono K, Katayama Y, Tamura T, Nishikawa M, Kugai N, Yamamoto M, Nagata N: The mouse mammary tumor cell line, MMT060562, produces prostaglandin E2 and leukemia inhibitory factor and supports osteoclast formation *in vitro* via a stromal cell-dependent pathway. J Bone Miner Res 13: 400–408, 1998
209. Mundy GR: Pathophysiology of cancer-associated hypercalcemia. Semin Oncol 17: 10–15, 1990
210. Roodman GD: Mechanisms of bone lesions in multiple myeloma and lymphoma. Cancer 80: 1557–1563, 1997
211. Thomas T, Lafage-Proust MH: Contribution of genetically modified mouse models to the elucidation of bone physiology. Rev Rhum Engl Ed 66: 728–735, 1999
212. Atkins GJ, Bouralexis S, Haynes DR, Graves SE, Geary SM, Evdokiou A, Zannettino AC, Hay S, Findlay DM: Osteoprotegerin inhibits osteoclast formation and bone resorbing activity in giant cell tumors of bone. Bone 28: 370–377, 2001
213. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD: Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. Blood 97: 3349–3353, 2001
214. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR: Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 98: 1544–1549, 1996
215. Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR, Yoneda T: Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 55: 3551–3557, 1995
216. Michigami T, Ihara-Watanabe M, Yamazaki M, Ozono K: Receptor activator of nuclear factor kappaB ligand (RANKL) is a key molecule of osteoclast formation for bone metastasis in a newly developed model of human neuroblastoma. Cancer Res 61: 1637–1644, 2001
217. Oyajobi BO, Anderson DM, Traianedes K, Williams PJ, Yoneda T, Mundy GR: Therapeutic efficacy of a soluble receptor activator of nuclear factor kappaB-IgG Fc fusion protein in suppressing bone resorption and hypercalcemia in a model of humoral hypercalcemia of malignancy. Cancer Res 61: 2572–2578, 2001
218. John A, Tuszynski G: The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathol Oncol Res 7: 14–23, 2001
219. Boag AH, Young ID: Immunohistochemical analysis of type IV collagenase expression in prostatic hyperplasia and adenocarcinoma. Mod Pathol 6: 65–68, 1993
220. Bodey B, Bodey B, Jr., Siegel SE, Kaiser HE: Immunocytochemical detection of matrix metalloproteinase expression in prostate cancer. *In vivo* 15: 65–70, 2001

221. Festuccia C, Bologna M, Vicentini C, Tacconelli A, Miano R, Violini S, Mackay AR: Increased matrix metalloproteinase-9 secretion in short-term tissue cultures of prostatic tumor cells. Int J Cancer 69: 386–393, 1996
222. Hamdy FC, Fadlon EJ, Cottam D, Lawry J, Thurrell W, Silcocks PB, Anderson JB, Williams JL, Rees RC: Matrix metalloproteinase 9 expression in primary human prostatic adenocarcinoma and benign prostatic hyperplasia. Br J Cancer 69: 177–182, 1994
223. Hashimoto K, Kihira Y, Matuo Y, Usui T: Expression of matrix metalloproteinase-7 and tissue inhibitor of metalloproteinase-1 in human prostate. J Urol 160: 1872–1876, 1998
224. Montironi R, Fabris G, Lucarini G, Biagini G: Location of 72-kd metalloproteinase (type IV collagenase) in untreated prostatic adenocarcinoma. Pathol Res Pract 191: 1140–1146, 1995
225. Montironi R, Lucarini G, Castaldini C, Galluzzi CM, Biagini G, Fabris G: Immunohistochemical evaluation of type IV collagenase (72-kd metalloproteinase) in prostatic intraepithelial neoplasia. Anticancer Res 16: 2057–2062, 1996
226. Pajouh MS, Nagle RB, Breathnach R, Finch JS, Brawer MK, Bowden GT: Expression of metalloproteinase genes in human prostate cancer. J Cancer Res Clin Oncol 117: 144–150, 1991
227. Duivenvoorden WC, Hirte HW, Singh G: Use of tetracycline as an inhibitor of matrix metalloproteinase activity secreted by human bone-metastasizing cancer cells. Invasion Metastasis 17: 312–322, 1997
228. Sanchez-Sweatman OH, Orr FW, Singh G: Human metastatic prostate PC3 cell lines degrade bone using matrix metalloproteinases. Invasion Metastasis 18: 297–305, 1998
229. Lee J, Weber M, Mejia S, Bone E, Watson P, Orr W: A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C nu/nu mice. Eur J Cancer 37: 106–113, 2001
230. Nemeth JA, Yousif R, Herzog M, Che M, Upadhyay J, Shekarriz B, Bhagat S, Mullins C, Fridman R, Cher ML: Matrix metalloproteinases activity, bone matrix turnover and tumor cell proliferation in prostate cancer bone metastasis. J Natl Cancer Inst 94: 17–25, 2002
231. Pirtskhalaishvili G, Nelson JB: Endothelium – derived factors as paracrine mediators of prostate cancer progression. Prostate 44: 77–87, 2000
232. Perkel VS, Mohan S, Baylink DJ, Linkhart TA: An inhibitory insulin-like growth factor binding protein (In-IGFBP) from human prostatic cell conditioned medium reveals N-terminal sequence identity with bone derived In-IGFBP. J Clin Endocrinol Metab 71: 533–535, 1990
233. Taguchi Y, Yamamoto M, Yamate T, Lin SC, Mocharla H, DeTogni P, Nakayama N, Boyce BF, Abe E, Manolagas SC: Interleukin-6-type cytokines stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage. Proc Assoc Am Physicians 110: 559–574, 1998
234. Le Brun G, Aubin P, Soliman H, Ropiquet F, Villette JM, Berthon P, Creminon C, Cussenot O, Fiet J: Upregulation of endothelin 1 and its precursor by IL-1beta, TNF-alpha, and TGF-beta in the PC3 human prostate cancer cell line. Cytokine 11: 157–162, 1999
235. Goltzman D, Karaplis AC, Kremer R, Rabbani SA: Molecular basis of the spectrum of skeletal complications of neoplasia. Cancer 88: 2903–2908, 2000

Address for offprints: Evan T. Keller, Room 5304 CCGCB, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0940 USA; *e-mail:* etkeller@umich.edu

Drugs of Today 2002, 38 (2): 91-102

CCC: 0025-7656/2002

THE ROLE OF OSTEOCLASTIC ACTIVITY IN PROSTATE CANCER SKELETAL METASTASES

Evan T. Keller

Unit for Laboratory Animal Medicine and Department of Pathology,
University of Michigan, Ann Arbor, Michigan, USA

CONTENTS

Summary 91
Introduction 92
Osteoclast biology 92
Receptor activator of nuclear factor-κB ligand 93
Matrix metalloproteinases 94
Parathyroid hormone-related protein 94
Interleukin-6 95
Therapy of cancer-associated osteolysis 95
Conclusions 96
Acknowledgments 96
References 96

Summary

Metastasis of prostate cancer to bone is a common complication of progressive prostate cancer. Skeletal metastases are often associated with severe pain and thus demand therapeutic interventions. Although often characterized as osteoblastic, prostate cancer skeletal metastases usually have an underlying osteoclastic component. Advances in osteoclast biology and pathophysiology have led toward defining putative therapeutic targets to attack tumor-induced osteolysis. Several factors have been found to be important in tumor-induced promotion of osteoclast activity. One key factor is the protein receptor activator of nuclear factor-κB ligand (RANKL), which is required to induce osteoclastogenesis. RANKL is produced by prostate cancer bone metastases, enabling these metastases to induce osteolysis through osteoclast activation. Another factor, osteoprotegerin, is a soluble decoy receptor for RANKL and inhibits RANKL-induced osteoclastogenesis. Osteoprotegerin has been shown in murine models to inhibit tumor-induced osteolysis. In addition to RANKL, parathyroid hormone-related protein and interleukin-6 are produced by prostate cancer cells and can promote osteoclastogenesis. Finally, matrix metalloproteinases (MMPs) are secreted by prostate cancer cells and promote osteolysis primarily through degradation of the nonmineralized bone matrix. MMP inhibitors have beer

Correspondence: Evan T. Keller, Room 5304, Comprehensive Cancer Geriatric Center, University of Michigan, 1500 E. Medical Center Dr., Ann Arbor, MI 48109-0940, USA. Tel: +1-734-615-0280; Fax: +1-734-936-9220; E-mail: etkeller@umich.edu

shown to diminish tumor establishment in bone in murine models. Thus, many factors derived from prostate cancer metastases can promote osteolysis, and these factors may serve as therapeutic targets.

The importance of osteoclasts in the establishment and progression of skeletal metastases has led to clinical evaluation of therapeutic agents to target them for slowing metastatic progression. Bisphosphonates are a class of compounds that decrease osteoclast life span by promoting their apoptosis. The bisphosphonate pamidronate has proven clinical efficacy for relieving bone pain associated with breast cancer metastases and has a promising outlook for prostate cancer metastases. Another bisphosphonate, zoledronic acid, appears to directly target prostate cancer cells in addition to diminishing osteoclast activity at the metastatic site. In addition to bisphosphonates, other novel therapies based on studies that delineate mechanisms of skeletal metastases establishment and progression will be developed in the near future.

Introduction

Prostate cancer metastasizes to bone in over 90% of men with progressive disease. Although primarily osteoblastic (*i.e.*, induce mineralization in the skeletal metastatic site), prostate skeletal metastases always have an underlying osteoclastic component. Tumor-induced osteolysis often results in severe pain and pathologic bone fractures and thus is an important target for prostate cancer therapy. Recent advances in the biology of osteoclasts provide clues to understanding the role of osteoclasts in cancer-induced bone lesions. Some of this research has led to clinical use of inhibitors of osteoclast activity to reduce tumor-induced osteolysis and bone pain. In this review, we will summarize the biology of osteoclasts, proosteoclastic factors produced by prostate cancer and therapeutic strategies designed to inhibit this painful aspect of cancer.

Osteoclast biology

Osteoclasts are derived from the colony-forming unit–granulocyte/macrophage hematopoietic precursor cells. The colony-forming unit-granulocyte/macrophage undergoes a defined progression of maturation steps that ultimately result in fusion of the precursor cells into mature osteoclasts (Fig. 1). Several factors promote osteoclastogenesis, including growth factors and cytokines. Both

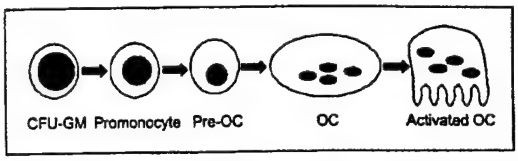

Fig. 1. Cellular pathway for osteoclastogenesis. Abbreviations: CFU-G/M, colony-forming unit-granulocyte/macrophage; OC, osteoclast.

colony-stimulating factor and interleukins-1 and -6 (IL-1 and IL-6) expand the osteoclast precursor pool. Tumor necrosis factor (TNF)-α promotes conversion of the promonocyte to a committed osteoclast precursor (1).

Although several factors promote osteoclastogenesis, one factor that is required for production of mature osteoclasts is receptor activator of nuclear factor-κB ligand (RANKL). A member of the TNF family, RANKL is initially expressed by bone marrow stromal cells, osteoblasts and activated T cells. RANKL is most commonly a membrane-anchored molecule; however, a small fraction of RANKL is released through proteolytic cleavage from the cell surface as a soluble 245-amino-acid homotrimeric molecule (2). Both soluble and membrane-bound RANKL promote osteoclast formation and activation by binding to RANK on the osteoclast precursor membrane (Fig. 2) (2-6) that has the characteristics of a monocyte (7). RANKL binding to RANK induces NF-κB and Fos activation (8, 9). Several lines of evidence demonstrate RANKL's importance in osteoclastogenesis. For example, RANKL has been shown to induce osteoclastogenesis *in vitro* from colony-forming unit-granulocyte/macrophage (10). Mice that are genetically engineered to overexpress RANKL or RANK are severely osteoporotic (11). Additionally, mice that have had their RANKL (12) or RANK (13) gene deleted have no osteoclasts and are osteopetrotic.

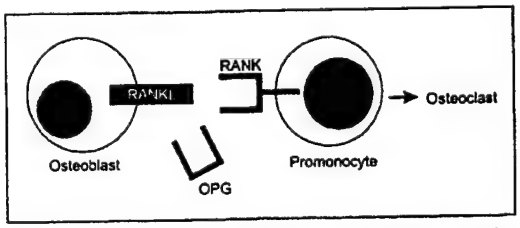

Fig. 2. RANKL and OPG regulation of osteoclastogenesis. Abbreviations: RANKL, receptor activator of nuclear factor-κB ligand.

In addition to RANKL and RANK, another key modulator of osteoclastogenesis is osteoprotegerin (also known as osteoclastogenesis inhibitory factor) (14, 15). Osteoprotegrin serves as a decoy receptor that binds RANKL and thus blocks its ability to bind to RANK and induce osteoclastogenesis. In contrast to RANKL and RANK, whose expression is mainly restricted at low levels to the skeletal and immune systems, osteoprotegrin is expressed in a variety of tissues, such as liver, lung, heart, kidney, stomach, intestines, skin and calvaria in mice, and lung, heart, kidney and placenta in humans (14, 16-21). In bone, osteoprotegrin is mainly produced by osteoblastic lineage cells and its expression increases as the cells become more differentiated (19, 22, 23). Several factors, including 1,25-dihydroxyvitamin D3, IL-1-β, TNF-α and BMP-2, induce osteoprotegrin mRNA expression in human osteoblast cell lines (19). Administration of recombinant osteoprotegrin to normal rodents resulted in increased bone mass (14, 17) and completely prevented ovariectomy-induced bone loss without apparent adverse skeletal and extraskeletal side effects (14). Additionally, a single subcutaneous injection of osteoprotegrin is effective in rapidly and profoundly reducing bone turnover for a sustained period in women (24). In fact, based on this activity, the balance ratio of RANKL to osteoprotegrin appears to be very important in controlling the overall activity (*i.e.*, lysis *vs.* no lysis) that will be observed (11, 23, 25, 26).

Once activated, osteoclasts resorb bone through secretion of a combination of proteases to resorb the nonmineralized matrix and acid to dissolve the hydroxyapatitic mineral (27). Proteases that are important mediators of osteoclastic activity include cathepsin K and metalloproteinases. Cathepsin K can cleave bone proteins such as type I collagen, osteopontin and osteonectin (28). Overexpression of cathepsin K in the mouse results in accelerated bone turnover (29), whereas knockout of cathepsin K results in retarded bone matrix degradation and osteopetrosis (30). Several novel classes of cathepsin K inhibitors have been designed and may provide novel therapeutic agents to target bone resorption (31, 32). In addition to the proteases, acid is secreted from osteoclasts to resorb the mineralized matrix. Acid is believed to be secreted through vacuolar H(+)-ATPase-dependent pumps present on the osteoclast's ruffled membranes (33). Several hormones regulate acid secretion, including parathyroid hormone, which increases acid secretion and calcitonin, which in turn decreases acid secretion. Carbonic anhydrase II appears to be an important mediator of acid production because acetazolamide, a carbonic-anhydrase- inhibitor-based diuretic, can block bone resorption (34). Another diuretic, indapamide, increased osteoblast proliferation and decreased bone resorption, at least in part, by decreasing osteoclast differentiation via a direct effect on hematopoietic precursors *in vitro* (35). These findings suggest that targeting osteoclast-derived activity, in addition to targeting osteoclast production or survival, may provide therapeutic avenues to diminish tumor-induced bone resorption.

Receptor activator of nuclear factor-κB ligand

As described above, RANKL is a key osteoclastogenic factor. Several lines of evidence support the role of RANKL in prostate cancer-mediated osteolysis. Although a bone metastatic prostate cancer cell line has been shown to express osteoprotegrin (36), that same line overexpresses RANKL (37). Additionally, in normal prostate, osteoprotegrin protein was detected in luminal epithelial and stromal cells (5%-65% and 15%-70%, respectively) and RANKL immunoreactivity was observed in 15%-50% of basal epithelial cells, 40%-90% of luminal epithelial cells and 70%-100% of stromal cells (38). Osteoprotegrin was not detected in 8 of 10 primary CaP specimens but RANKL was heterogeneously expressed in 10 of 11 CaP specimens (38). Importantly, the percentage of tumor cells expressing osteoprotegrin and RANKL was significantly increased in all CaP bone metastases compared with nonosseous metastases or primary CaP. Serum osteoprotegrin levels are elevated in patients with advanced prostate cancer compared with less advanced prostate cancer (39). However, RANKL levels were not measured in that study, thus one cannot determine if the ratio of RANKL: osteoprotegrin was altered in these patients. It is possible that RANKL is only expressed locally at the skeletal metastatic site and therefore not detectable in the serum. Regardless, taken together, these observations suggest that the RANKL:osteoprotegrin axis may play an important role in prostate cancer bone metastases. Further support for this possibility was demonstrated by the observation that administration of osteoprotegrin prevented establishment of prostate cancer cells in the bones of SCID mice, although it had no effect on establishment of subcutaneous tumors in the same mice (37).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs), a family of enzymes whose primary function is to degrade the extracellular matrix, play a role in bone remodeling. This activity occurs in the absence of osteoclasts (40), suggesting that MMPs have a direct resorptive effect. Several have the ability to degrade the non-mineralized matrix of bone including MMP-1, MMP-9 and MMP-13, which are collagenases. Other MMPs, such as stromelysin (MMP-3), activate MMP-1. Through their proteolytic activity MMPs contribute to metastatic invasion, including destruction of bone (41).

Prostate carcinomas and their cell lines express a large number of MMPs (42-49). Levels of MMP-9 secretion in primary prostate cancer cultures increased with Gleason histological grade (44). Active MMP-9 species were detected in 15 cultures (31%) of primary prostate cancer tissues. The presence of the mineralized matrix has been shown to induce MMP-9 expression from prostate carcinoma cells (50).

The initial functional data that suggested prostate carcinoma bone metastasis modulated bone remodeling through MMPs was provided by *in vitro* studies. Specifically, blocking MMP activity with 1,10-phenanthroline, an MMP inhibitor, diminished bone matrix degradation induced by PC-3 cells *in vitro* (51, 52). Matrilysin (MMP-7) has been shown to be up-regulated in DU-145 prostate cancer cells and can enhance their invasive ability. Monoclonal antibody targeting the cytokine interleukin-6 (IL-6) has been shown to increase promatrilysin expression in DU-145 cultures (53). This suggests that IL-6, which is increased in prostate cancer (reviewed in 54), enhances prostate cancer invasion through production of MMP-7.

The importance of MMPs in bone metastasis has been further confirmed *in vivo*. An MMP inhibitor, batimastat, has been shown to inhibit development of bone resorption *in vitro* and *in vivo* in murine models of breast (55) and prostate carcinoma (56). The mechanism through which prostate-carcinoma-produced MMPs induce bone resorption is not clear; however, it appears to involve induction of osteoclastogenesis, as inhibition of MMPs reduced the number of osteoclasts associated with prostate tumor growth in human bone implants in mice (56). Additionally, the bisphosphonate alendronate blocked MMP production from PC-3 cells (57). This was associated with diminished establishment of bone metastasis in mice injected with PC-3 tumors (40).

Parathyroid hormone-related protein

Parathyroid hormone-related protein (PTHrP), a protein with limited homology to parathyroid hormone, was originally identified as a tumor-derived factor responsible for humoral hypercalcemia of malignancy. Parathyroid hormone and PTHrP bind to the same receptor (the parathyroid hormone-1 receptor) and evoke the same biological activity due to similarities in their steric configurations at the region of 25-34 amino acids. Patients with solid tumors and hypercalcemia have increased serum PTHrP in 80% of the cases, emphasizing the impact of this peptide to increase bone resorption and renal tubular resorption of calcium (58). Subsequent to its characterization in humoral hypercalcemia of malignancy, PTHrP was found to be produced by many normal tissues, including epithelium, lactating mammary gland and cartilage, where it has an autocrine, paracrine or intracrine role (58).

PTHrP is an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid (59, 60). PTHrP has been immunohistochemically identified in prostate carcinoma tissue in patients with clinically localized disease (61), is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium, is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia (62, 63) and is found in human metastatic lesions in bone (64). However, in some studies, expression of PTHrP receptor in prostate cancer appears to be more consistent than expression of PTHrP itself (65). Overexpression of ras oncogene in immortalized prostate epithelial cells has been shown to promote PTHrP expression (66). This may account for the increased expression of PTHrP as the cells progress to a malignant phenotype.

There is evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma (67), enhance breast cancer metastasis to bone (68, 69) and act as an autocrine growth factor for prostate carcinoma cells *in vitro* (59), although it does not effect proliferation of normal prostate cells (70). Recent evidence indicates that expression of nuclear-targeted PTHrP can protect prostate and other cells from apoptosis (64, 71), bind RNA (72) and act as a mitogen (73, 74). PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model (64), suggesting that

PTHrP acts in an autocrine or intracrine mechanism to promote tumor growth. In contrast, in this same model and in an intracardiac injection model of prostate carcinoma, PTHrP was not associated with an increase in metastatic potential (64, 75). This suggests that PTHrP is not important in the process of metastasis to bone, but once in the bone microenvironment where target cells with receptors are present (osteoblasts), it may play a critical role in the bone response to prostate carcinoma. Of particular interest to prostate carcinoma, prostate specific antigen has been shown to cleave PTHrP leading to an inactivation of the PTHrP-stimulation of cAMP, which is a key pathway for the actions of PTHrP in bone (76). Overexpression of PTHrP in prostate cancer cells has been shown to induce osteolytic lesions in the bone of rats (77), although the level of expression may not directly correlate with the degree of osteolysis (75). All these data suggest that PTHrP has a critical role in the local bone microenvironment of metastatic prostate carcinoma, but this precise role is yet to be determined.

Interleukin-6

IL-6 belongs to the "interleukin-6-type cytokine" family that also includes leukemia inhibitory factor, interleukin-11, ciliary neurotrophic factor, cardiotrophin-1 and oncostatin M (78). Many physiologic functions are attributed to IL-6, including promotion of antibody production from B lymphocytes, modulation of hepatic acute-phase reactant synthesis, promotion of osteoclastic mediated bone resorption and induction of thrombopoiesis (79). IL-6 mediates its activity through the IL-6 receptor complex, which is composed of two components: an 80 Kd transmembrane receptor (IL-6Rp80, IL-6R, α-subunit) that specifically binds IL-6 but has no signaling capability and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6R binding (80). In addition to the transmembrane IL-6R, a soluble form of IL-6R exists that is produced by either proteolytic cleavage of the 80 kDa subunit (81, 82) or differential splicing of mRNA (83). Although the soluble IL-6R does not possess a transmembrane component, it can still bind to IL-6, and the ligand-bound soluble IL-6R•IL-6 complex activates signal transduction and biological responses through membrane-bound gp130 (84).

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer (85-87). Adler *et al.* (85) demonstrated that serum levels of IL-6 and transforming growth factor-β1 are elevated in patients with metastatic prostate cancer and that these levels correlate with tumor burden as assessed by serum prostate-specific antigen or clinically evident metastases. In a similar fashion, Drachenberg *et al.* (88) reported elevated serum IL-6 levels in men with hormone-refractory prostate cancer compared with normal controls, benign prostatic hyperplasia, prostatitis and localized or recurrent disease. In an animal model, prostate tumor cells injected next to human bones implanted in the limb of mice demonstrated IL-6 expression (89). In addition to IL-6, the IL-6R has been identified in human normal prostate and prostate carcinoma tissue (90, 91).

The secretion of IL-6 by prostate cancer cells in the bone microenvironment may impact bone remodeling (reviewed in 92, 93). IL-6 promotes osteoclastogenesis (94-96) most likely through increasing osteoclastogenic precursors. IL-6-mediated osteoclastogenesis is directly related to the level of gp130 present on the precursor cells (97). It appears that IL-6-mediated osteoclastogenesis is independent of promoting RANKL expression (98). However, IL-6 has been shown to potentiate PTHrP-induced osteoclastogenesis (99, 100). Administration of anti-IL-6 antibody has been shown to diminish growth of subcutaneously injected prostate cancer cells in nude mice, thus demonstrating the potential utility of this compound in clinical prostate cancer (101). These results strongly suggest that IL-6 may serve as a therapeutic target for the osteolytic component of prostate cancer skeletal metastases.

Therapy of cancer-associated osteolysis

Bone metastases are associated with several clinical sequelae, including bone pain, neuralgia, pathologic bone fracture and myelophthisis. Thus, targeting these lesions has received much research effort. Bisphosphonates are a group of chemicals that inhibit osteoclast activity resulting in decreased bone resorption and thus have received much attention as inhibitors of clinical complications of bone metastases (102-104). Bisphosphonates work directly on osteoclasts to induce their apoptosis (105, 106). Animal studies have demonstrated that bisphosphonates can diminish tumor-induced osteoclastogenesis and osteolysis (107-111); although in some instances it appears to only reduce tumor-induced lysis but not tumor burden (112). Studies in breast cancer and myeloma patients have shown that these agents markedly inhibit the progression of bone disease, resulting in improved survival and decreased

morbidity from bone pain and fracture (113, 114). These results have led to their incorporation into standard treatment regimens for skeletal metastases associated with these cancers.

In addition to inhibiting osteoclast survival, bisphosphonates may have direct effects on tumor cells (115). For example, several bisphosphonates induce apoptosis in myeloma cells (116-118). However, this is not the case for all bisphosphonates (119). In addition to inducing apoptosis, bisphosphonates have been shown to inhibit breast carcinoma cell adhesion to bone (120). Furthermore, alendronate blocked collagen degradation and MMP release from prostate cancer cells (57, 121). Taken together, these findings suggest that bisphosphonate action is not limited to inhibition of osteoclasts.

Studies of bisphosphonates use in patients with prostate cancer skeletal metastases have generally shown a decrease in bone pain, although some studies have shown no benefit (122-124). A recent randomized study of the oral bisphosphonate clodronate showed an encouraging decrease in the rate of progression to symptomatic bone metastases in men with prostate cancer (125). Consistent with this observation is the finding that zoledronic acid, a third generation bisphosphonate, has demonstrated significantly increased activity in preclinical models when compared with early agents in this class. Exposure of prostate cancer cell lines to zoledronic acid results in marked inhibition of cell proliferation, suggesting that this agent may have a direct antitumor effect beyond its ability to inhibit osteoclast activity (126, 127). Zoledronic acid also has been shown to inhibit the invasion of prostate carcinoma cell lines *in vitro* (128). Clinical studies have demonstrated efficacy in treating humoral hypercalcemia of malignancy, leading to recent U.S. FDA approval for use in this clinical setting (129). Treatment with zoledronic acid results in a significant and sustained decrease in markers of bone metabolism.

Conclusions

Prostate cancer skeletal metastases promote osteolysis through several mechanisms that include both activation of osteoclast-mediated bone resorption and direct resorption on nonmineralized bone matrix (Fig. 3). Delineating the mechanisms that promote prostate cancer skeletal metastasis and the interactions between metastatic prostate cancer cells and bones should lead to development of therapies that will diminish or prevent these events. Our current understanding of the biology of prostate cancer skeletal metastases has led to identification of several putative targets and therapies aimed at these targets, some of which are currently in clinical trials at the time of this writing. Continued research into the biology of prostate cancer skeletal metastases should enable development of improved therapeutic regimens to diminish this painful aspect of prostate cancer.

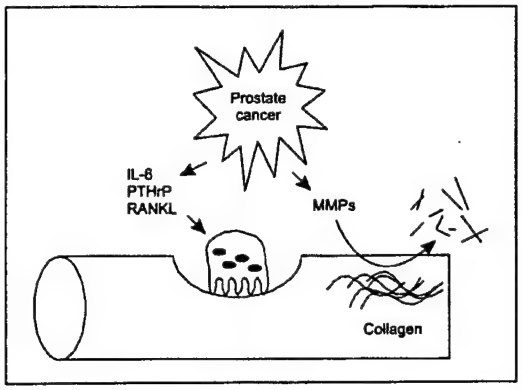

Fig. 3. Mechanisms of prostate cancer metastases-mediated osteolysis. Abbreviations: IL-6, interleukin-6; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator of nuclear factor-κB ligand; MMPs, matrix metalloproteinases.

Acknowledgments

This work was supported, in part, by USAMRMC Prostate Carcinoma Research Program Grant # DAMD17-00-1-0530, CaP CURE research award and National Institutes of Health Grants SPORE 1 P50 CA69568

References

1. Uy, H.L., Mundy, G.R., Boyce, B.F. et al. *Tumor necrosis factor enhances parathyroid hormone-related protein-induced hypercalcemia and bone resorption without inhibiting bone formation in vivo.* Cancer Res 1997, 57: 3194-9.
2. Lum, L., Wong, B.R., Josien, R. et al. *Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival.* J Biol Chem 1999, 274: 13613-8.
3. Lacey, D.L., Timms, E., Tan, H.L. et al. *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* Cell 1998, 93: 165-76.

4. Kong, Y.Y., Yoshida, H., Sarosi, I. et al. *OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis.* Nature 1999, 397: 315-23.
5. Yasuda, H., Shima, N., Nakagawa, N. et al. *Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL.* Proc Natl Acad Sci USA 1998, 95: 3597-602.
6. Fuller, K., Wong, B., Fox, S., Choi, Y., Chambers, T.J. *TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts.* J Exp Med 1998, 188: 997-1001.
7. Shalhoub, V., Elliott, G., Chiu, L. et al. *Characterization of osteoclast precursors in human blood.* Br J Haematol 2000, 111: 501-12.
8. Matsuo, K., Owens, J.M., Tonko, M., Elliott, C., Chambers, T.J., Wagner, E.F. *Fos11 is a transcriptional target of c-Fos during osteoclast differentiation.* Nat Genet 2000, 24: 184-7.
9. Hofbauer, L.C., Heufelder, A.E. *The role of osteoprotegerin and receptor activator of nuclear factor kappaB ligand in the pathogenesis and treatment of rheumatoid arthritis.* Arthritis Rheum 2001, 44: 253-9.
10. Menaa, C., Kurihara, N., Roodman, G.D. *CFU-GM-derived cells form osteoclasts at a very high efficiency.* Biochem Biophys Res Commun 2000, 267: 943-6.
11. Hofbauer, L.C., Neubauer, A., Heufelder, A.E. *Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: Potential implications for the pathogenesis and treatment of malignant bone diseases.* Cancer 2001, 92: 460-70.
12. Pettit, A.R., Ji, H., von Stechow, D. et al. *TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis.* Am J Pathol 2001, 159: 1689-99.
13. Li, J., Sarosi, I., Yan, X.Q. et al. *RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism.* Proc Natl Acad Sci USA 2000, 97: 1566-71.
14. Simonet, W.S., Lacey, D.L., Dunstan, C.R. et al. *Osteoprotegerin: A novel secreted protein involved in the regulation of bone density.* Cell 1997, 89: 309-19.
15. Tsuda, E., Goto, M., Mochizuki, S. et al. *Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis.* Biochem Biophys Res Commun 1997, 234: 137-42.
16. Tan, K.B., Harrop, J., Reddy, M. et al. *Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells.* Gene 1997, 204: 35-46.
17. Yasuda, H., Shima, N., Nakagawa, N. et al. *Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): A mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro.* Endocrinology 1998, 139: 1329-37.
18. Yun, T.J., Chaudhary, P.M., Shu, G.L. et al. *OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40.* J Immunol 1998, 161: 6113-21.
19. Hofbauer, L.C., Dunstan, C.R., Spelsberg, T.C., Riggs, B.L., Khosla, S. *Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines.* Biochem Biophys Res Commun 1998, 250: 776-81.
20. Hofbauer, L.C., Heufelder A.E. *Osteoprotegerin and its cognate ligand: A new paradigm of osteoclastogenesis.* Eur J Endocrinol 1998, 139: 152-4.
21. Vidal, N.O., Brandstrom, H., Jonsson, K.B., Ohlsson, C. *Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: Down-regulation by glucocorticoids.* J Endocrinol 1998, 159: 191-5.
22. Kartsogiannis, V., Zhou, H., Horwood, N.J. et al. *Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues.* Bone 1999, 25: 525-34.
23. Nagai, M., Sato, N. *Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation.* Biochem Biophys Res Commun 1999, 257: 719-23.
24. Bekker, P.J., Holloway, D., Nakanishi, A., Arrighi, M., Leese, P.T., Dunstan, C.R. *The effect of a single dose of osteoprotegerin in postmenopausal women.* J Bone Miner Res 2001, 16: 348-60.
25. Thomas, G.P., Baker, S.U., Eisman, J.A., Gardiner, E.M. *Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts.* J Endocrinol 2001, 170: 451-60.
26. Fazzalari, N.L., Kuliwaba, J.S., Atkins, G.J., Forwood, M.R., Findlay, D.M. *The ratio of mes-*

senger RNA levels of receptor activator of nuclear factor kappa B ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. J Bone Miner Res 2001, 16: 1015-27.

27. Blair, H.C. *How the osteoclast degrades bone.* Bioessays 1998, 20: 837-46.
28. Katunuma, N. *Mechanism and regulation of bone resorption by osteoclasts.* Curr Top Cell Regul 1997, 35: 179-92.
29. Kiviranta, R., Morko, J., Uusitalo, H., Aro, H.T., Vuorio, E., Rantakokko, J. *Accelerated turnover of metaphyseal trabecular bone in mice overexpressing cathepsin K.* J Bone Miner Res 2001, 16: 1444-52.
30. Gowen, M., Lazner, F., Dodds, R. et al. *Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization.* J Bone Miner Res 1999, 14: 1654-63.
31. Yamashita, D.S., Dodds, R.A. *Cathepsin K and the design of inhibitors of cathepsin K.* Curr Pharm Des 2000, 6: 1-24.
32. Stroup, G.B., Lark, M.W., Veber, D.F. et al. *Potent and selective inhibition of human cathepsin K leads to inhibition of bone resorption in vivo in a nonhuman primate.* J Bone Miner Res 2001, 16: 1739-46.
33. Lee, B.S., Holliday, L.S., Ojikutu, B., Krits, I., Gluck, S.L. *Osteoclasts express the B2 isoform of vacuolar H(+)-ATPase intracellularly and on their plasma membranes.* Am J Physiol 1996, 270: C382-8.
34. Lehenkari, P., Hentunen, T.A., Laitala-Leinonen, T., Tuukkanen, J., Vaananen, H.K. *Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca^{2+}.* Exp Cell Res 1998, 242: 128-37.
35. Lalande, A., Roux, S., Denne, M.A. et al. *Indapamide, a thiazide-like diuretic, decreases bone resorption in vitro.* J Bone Miner Res 2001, 16: 361-70.
36. Lin, D.L., Tarnowski, C.P., Zhang, J. et al. *Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro.* Prostate 2001, 47: 212-21.
37. Zhang, J., Dai, J., Qi, Y. et al. *Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone.* J Clin Invest 2001, 107: 1235-44.
38. Brown, J.M., Corey, E., Lee, Z.D. et al. *Osteoprotegerin and rank ligand expression in prostate cancer.* Urology 2001, 57: 611-6.
39. Brown, J.M., Vessella, R.L., Kostenuik, P.J., Dunstan, C.R., Lange, P.H., Corey, E. *Serum osteoprotegerin levels are increased in patients with advanced prostate cancer.* Clin Cancer Res 2001, 7: 2977-83.
40. Stearns, M.E., Wang, M. *Effects of alendronate and taxol on PC-3 ML cell bone metastases in SCID mice.* Invasion Metastasis 1996, 16: 116-31.
41. John, A., Tuszynski, G. *The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis.* Pathol Oncol Res 2001, 7: 14-23.
42. Boag, A.H., Young, I.D. *Immunohistochemical analysis of type IV collagenase expression in prostatic hyperplasia and adenocarcinoma.* Mod Pathol 1993, 6: 65-8.
43. Bodey, B., Bodey, B., Jr., Siegel, S.E., Kaiser, H.E. *Immunocytochemical detection of matrix metalloproteinase expression in prostate cancer.* In Vivo 2001, 15: 65-70.
44. Festuccia, C., Bologna, M., Vicentini, C. et al. *Increased matrix metalloproteinase-9 secretion in short-term tissue cultures of prostatic tumor cells.* Int J Cancer 1996, 69: 386-93.
45. Hamdy, F.C., Fadlon, E.J., Cottam, D. et al. *Matrix metalloproteinase 9 expression in primary human prostatic adenocarcinoma and benign prostatic hyperplasia.* Br J Cancer 1994, 69: 177-82.
46. Hashimoto, K., Kihira, Y., Matuo, Y., Usui, T. *Expression of matrix metalloproteinase-7 and tissue inhibitor of metalloproteinase-1 in human prostate.* J Urol 1998, 160: 1872-6.
47. Montironi, R., Fabris, G., Lucarini, G., Biagini, G. *Location of 72-kd metalloproteinase (type IV collagenase) in untreated prostatic adenocarcinoma.* Pathol Res Pract 1995, 191: 1140-6.
48. Montironi, R., Lucarini, G., Castaldini, C., Galluzzi, C.M., Biagini, G., Fabris, G. *Immunohistochemical evaluation of type IV collagenase (72-kd metalloproteinase) in prostatic intraepithelial neoplasia.* Anticancer Res 1996, 16: 2057-62.
49. Pajouh, M.S., Nagle, R.B., Breathnach, R., Finch, J.S., Brawer, M.K., Bowden, G.T. *Expression of metalloproteinase genes in human prostate cancer.* J Cancer Res Clin Oncol 1991, 117: 144-50.
50. Festuccia, C., Giunciuglio, D., Guerra, F. et al. *Osteoblasts modulate secretion of urokinase-type plasminogen activator (uPA) and matrix*

metalloproteinase-9 (MMP-9) in human prostate cancer cells promoting migration and matrigel invasion. Oncol Res 1999, 11: 17-31.

51. Duivenvoorden, W.C., Hirte, H.W., Singh, G. *Use of tetracycline as an inhibitor of matrix metalloproteinase activity secreted by human bone-metastasizing cancer cells.* Invasion Metastasis 1997, 17: 312-22.
52. Sanchez-Sweatman, O.H., Orr, F.W., Singh, G. *Human metastatic prostate PC3 cell lines degrade bone using matrix metalloproteinases.* Invasion Metastasis 1998, 18: 297-305.
53. Stratton, M.S., Sirvent, H., Udayakumar, T.S., Nagle, R.B., Bowden, G.T. *Expression of the matrix metalloproteinase promatrilysin in coculture of prostate carcinoma cell lines.* Prostate 2001, 48: 206-9.
54. Smith, P.C., Hobish, A., Lin, D., Culig, Z., Keller, E.T. *Interleukin-6 and prostate cancer progression.* Cytokine Growth Factor Rev 2001, 12: 33-40.
55. Lee, J., Weber, M., Mejia, S., Bone, E., Watson, P., Orr, W. *A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C nu/nu mice.* Eur J Cancer 2001, 37: 106-13.
56. Nemeth, J.A., Yousif, R., Herzog, M. et al. *Matrix metalloproteinases, bone matrix turnover and tumor cell proliferation in prostate cancer bone metastasis.* J Natl Cancer Inst 2002, 94: 17-25.
57. Stearns, M.E., Wang, M. *Alendronate blocks metalloproteinase secretion and bone collagen I release by PC-3 ML cells in SCID mice.* Clin Exp Metastasis 1998, 16: 693-702.
58. Strewler, G.J. *The physiology of parathyroid hormone-related protein.* N Engl J Med 2000, 342: 177-85.
59. Iwamura, M., Abrahamsson, P.A., Foss, K.A., Wu, G., Cockett, A.T., Deftos, L.J. *Parathyroid hormone-related protein: A potential autocrine growth regulator in human prostate cancer cell lines.* Urology 1994, 43: 675-9.
60. Deftos, L.J. *Prostate carcinoma: Production of bioactive factors.* Cancer 2000, 88: 3002-8.
61. Iwamura, M., di Sant'Agnese, P.A., Wu, G. et al. *Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer.* Cancer Res 1993, 53: 1724-6.
62. Asadi, F., Farraj, M., Sharifi, R., Malakouti, S., Antar, S., Kukreja, S. *Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia.* Hum Pathol 1996, 27: 1319-23.
63. Iwamura, M., Gershagen, S., Lapets, O. et al. *Immunohistochemical localization of parathyroid hormone-related protein in prostatic intraepithelial neoplasia.* Hum Pathol 1995, 26: 797-801.
64. Dougherty, K.M., Blomme, E.A., Koh, A.J. et al. *Parathyroid hormone-related protein as a growth regulator of prostate carcinoma.* Cancer Res 1999, 59: 6015-22.
65. Iddon, J., Bundred, N.J., Hoyland, J. et al. *Expression of parathyroid hormone-related protein and its receptor in bone metastases from prostate cancer.* J Pathol 2000, 191: 170-4.
66. Kremer, R., Goltzman, D., Amizuka, N., Webber, M.M., Rhim, J.S. *ras Activation of human prostate epithelial cells induces overexpression of parathyroid hormone-related peptide.* Clin Cancer Res 1997, 3: 855-9.
67. Burton, P.B., Moniz, C., Knight, D.E. *Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma.* Biochem Biophys Res Commun 1990, 167: 1134-8.
68. Bouizar, Z., Spyratos, F., de Vernejoul, M.C. *The parathyroid hormone-related protein (PTHrP) gene: Use of downstream TATA promotor and PTHrP 1-139 coding pathways in primary breast cancers vary with the occurrence of bone metastasis.* J Bone Miner Res 1999, 14: 406-14.
69. Yin, J.J., Selander, K., Chirgwin, J.M. et al. *TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development.* J Clin Invest 1999, 103: 197-206.
70. Peehl, D.M., Edgar, M.G., Cramer, S.D., Deftos, L.J. *Parathyroid hormone-related protein is not an autocrine growth factor for normal prostatic epithelial cells.* Prostate 1997, 31: 47-52.
71. Henderson, J.E., Amizuka, N., Warshawsky, H. et al. *Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death.* Mol Cell Biol 1995, 15: 4064-75.
72. Aarts, M.M., Rix, A., Guo, J., Bringhurst, R., Henderson, J.E. *The nucleolar targeting signal (NTS) of parathyroid hormone related protein mediates endocytosis and nucleolar translocation.* J Bone Miner Res 1999, 14: 1493-503.

73. Ye, Y., Wang, C., Du, P., Falzon, M., Seitz, P.K., Cooper, C.W. *Overexpression of parathyroid hormone-related protein enhances apoptosis in the rat intestinal cell line, IEC-6.* Endocrinology 2001, 142: 1906-14.
74. Massfelder, T., Dann, P., Wu, T.L., Vasavada, R., Helwig, J.J., Stewart, A.F. *Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: A critical role for nuclear targeting.* Proc Natl Acad Sci USA 1997, 94: 13630-5.
75. Blomme, E.A., Dougherty, K.M., Pienta, K.J., Capen, C.C., Rosol, T.J., McCauley, L.K. *Skeletal metastasis of prostate adenocarcinoma in rats: Morphometric analysis and role of parathyroid hormone-related protein.* Prostate 1999, 39: 187-97.
76. Cramer, S.D., Chen, Z., Peehl, D.M. *Prostate-specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: Inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts.* J Urol 1996, 156: 526-31.
77. Rabbani, S.A., Gladu, J., Harakidas, P., Jamison, B., Goltzman, D. *Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo.* Int J Cancer 1999, 80: 257-64.
78. Sehgal, P., Wang, L., Rayanade, R., Pan, H., Margulies, L. *Interleukin-6-type cytokines.* Ann NY Acad Sci 1995, 762: 1-14.
79. Hirano, T. *The biology of interleukin-6.* Chem Immunol 1992, 51: 153-80.
80. Taga, T., Hibi, M., Murakami, M. et al. *Interleukin-6 receptor and signals.* Chem Immunol 1992, 51: 181-204.
81. Mullberg, J., Oberthur, W., Lottspeich, F. et al. *The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site.* J Immunol 1994, 152: 4958-68.
82. Rose-John, S., Heinrich, P.C. *Soluble receptors for cytokines and growth factors: Generation and biological function.* Biochem J 1994, 300: 281-90.
83. Lust, J.A., Donovan, K.A., Kline, M.P., Greipp, P.R., Kyle, R.A., Maihle, N.J. *Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor.* Cytokine 1992, 4: 96-100.
84. Mackiewicz, A., Schooltink, H., Heinrich, P.C., Rose-John, S. *Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins.* J Immunol 1992, 149: 2021-7.
85. Adler, H.L., McCurdy, M.A., Kattan, M.W., Timme, T.L., Scardino, P.T., Thompson, T.C. *Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma.* J Urol 1999, 161: 182-7.
86. Hoosien, N., Abdul, M., McCabe, R. et al. *Clinical significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostate cancer.* Urol Oncol 1995, 1: 246-51.
87. Twillie, D.A., Eisenberger, M.A., Carducci, M.A., Hseih, W.S., Kim, W.Y., Simons, J.W. *Interleukin-6: A candidate mediator of human prostate cancer morbidity.* Urology 1995, 45: 542-9.
88. Drachenberg, D.E., Elgamal, A.A., Rowbotham, R., Peterson, M., Murphy, G.P. *Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer.* Prostate 1999, 41: 127-33.
89. Tsingotjidou, A.S., Zotalis, G., Jackson, K.R. et al. *Development of an animal model for prostate cancer cell metastasis to adult human bone.* Anticancer Res 2001, 21: 971-8.
90. Siegsmund, M.J., Yamazaki, H., Pastan, I. *Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia.* J Urol 1994, 151: 1396-9.
91. Hobisch, A., Rogatsch, H., Hittmair, A. et al. *Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue.* J Pathol 2000, 191: 239-44.
92. Ershler, W.B., Keller, E.T. *Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty.* Annu Rev Med 2000, 51: 245-70.
93. Manolagas, S.C. *The role of IL-6 type cytokines and their receptors in bone.* Ann NY Acad Sci 1998, 840: 194-204.
94. Jilka, R.L., Passeri, G., Girasole, G. et al. *Estrogen loss upregulates hematopoiesis in the mouse: A mediating role of IL-6.* Exp Hematol 1995, 23: 500-6.
95. Poli, V., Balena, R., Fattori, E. et al. *Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion.* EMBO J 1994, 13: 1189-96.
96. de Grooth, R., Kawilarang-de Haas, E.W., van de Sande-Rijkers, C.M., Nijweide, P.J. *The role of osteoblast density and endogenous interleukin-6 production in osteoclast formation from the hemopoietic stem cell line FDCP-MIX C2GM in coculture with primary osteoblasts.* Calcif Tissue Int 1998, 63: 57-62.

97. O'Brien, C.A., Lin, S.C., Bellido, T., Manolagas, S.C. *Expression levels of gp130 in bone marrow stromal cells determine the magnitude of osteoclastogenic signals generated by IL-6-type cytokines.* J Cell Biochem 2000, 79: 532-41.
98. Hofbauer, L.C., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C., Riggs, B.L., Khosla, S. *Interleukin-1 beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells.* Bone 1999, 25: 255-9.
99. Greenfield, E.M., Shaw, S.M., Gornik, S.A., Banks, M.A. *Adenyl cyclase and interleukin 6 are downstream effectors of parathyroid hormone resulting in stimulation of bone resorption.* J Clin Invest 1995, 96: 1238-44.
100. de la Mata, J., Uy, H.L., Guise, T.A. et al. *Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo.* J Clin Invest 1995, 95: 2846-52.
101. Smith, P.C., Keller, E.T. *Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice.* Prostate 2001, 48: 47-53.
102. Major, P.P., Lipton, A., Berenson, J., Hortobagyi, G. *Oral bisphosphonates: A review of clinical use in patients with bone metastases.* Cancer 2000, 88: 6-14.
103. Mundy, G.R. *Bisphosphonates as anticancer drugs.* Expert Opin Investig Drugs 1999, 8: 2009-15.
104. Diel, I.J., Solomayer, E., Bastert, G. *Bisphosphonates and the prevention of metastasis.* Cancer 2000, 88: 3080-8.
105. Rowe, D.J., Etre, L.A., Lovdahl, M.J., Pietrzyk, D.J. *Relationship between bisphosphonate concentration and osteoclast activity and viability.* In Vitro Cell Dev Biol Anim 1999, 35: 383-8.
106. Fleisch, H. *Mechanisms of action of the bisphosphonates.* Medicina (B Aires) 1997, 57 (Suppl. 1): 65-75.
107. Clohisy, D.R., O'Keefe, P.F., Ramnaraine, M.L. *Pamidronate decreases tumor-induced osteoclastogenesis in osteopetrotic mice.* J Orthop Res 2001, 19: 554-8.
108. Kurth, A.H., Kim, S.Z., Sedlmeyer, I., Hovy, L., Bauss, F. *Treatment with ibandronate preserves bone in experimental tumour-induced bone loss.* J Bone Joint Surg Br 2000, 82: 126-30.
109. Yoneda, T., Michigami, T., Yi, B., Williams, P.J., Niewolna, M., Hiraga, T. *Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma.* Cancer 2000, 88: 2979-88.
110. Hall, D.G., Stoica, G. *Effect of the bisphosphonate risedronate on bone metastases in a rat mammary adenocarcinoma model system.* J Bone Miner Res 1994, 9: 221-30.
111. Yoneda, T., Sasaki, A., Dunstan, C. et al. *Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate ibandronate and tissue inhibitor of the matrix metalloproteinase-2.* J Clin Invest 1997, 99: 2509-17.
112. Dallas, S.L., Garrett, I.R., Oyajobi, B.O. et al. *Ibandronate reduces osteolytic lesions but not tumor burden in a murine model of myeloma bone disease.* Blood 1999, 93: 1697-706.
113. Lipton, A. *Bisphosphonates and breast carcinoma: Present and future.* Cancer 2000, 88: 3033-7.
114. Apperley, J.F., Croucher, P.I. *Bisphosphonates in multiple myeloma.* Pathol Biol (Paris) 1999, 47: 178-81.
115. Shipman, C.M., Rogers, M.J., Apperley, J.F., Graham, R., Russell, G., Croucher, P.I. *Antitumour activity of bisphosphonates in human myeloma cells.* Leuk Lymphoma 1998, 32: 129-38.
116. Shipman, C.M., Croucher, P.I., Russell, R.G., Helfrich, M.H., Rogers, M.J. *The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway.* Cancer Res 1998, 58: 5294-7.
117. Takahashi, R., Shimazaki, C., Inaba, T. et al. *A newly developed bisphosphonate, YM529, is a potent apoptosis inducer of human myeloma cells.* Leuk Res 2001, 25: 77-83.
118. Aparicio, A., Gardner, A., Tu, Y., Savage, A., Berenson, J., Lichtenstein, A. *In vitro cytoreductive effects on multiple myeloma cells induced by bisphosphonates.* Leukemia 1998, 12: 220-9.
119. Shipman, C.M., Vanderkerken, K., Rogers, M.J. et al. *The potent bisphosphonate ibandronate does not induce myeloma cell apoptosis in a murine model of established multiple myeloma.* Br J Haematol 2000, 111: 283-6.
120. Magnetto, S., Boissier, S., Delmas, P.D., Clezardin, P. *Additive antitumor activities of tax-*

oids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. Int J Cancer 1999, 83: 263-9.

121. Steams, M.E. *Alendronate blocks TGF-beta1 stimulated collagen 1 degradation by human prostate PC-3 ML cells.* Clin Exp Metastasis 1998, 16: 332-9.
122. Pelger, R.C., Hamdy, N.A., Zwinderman, A.H., Lycklama a Nijeholt, A.A., Papapoulos, S.E. *Effects of the bisphosphonate olpadronate in patients with carcinoma of the prostate metastatic to the skeleton.* Bone 1998, 22: 403-8.
123. Harvey, H.A., Lipton, A. *The role of bisphosphonates in the treatment of bone metastases – The U.S. experience.* Support Care Cancer 1996, 4: 213-7.
124. Heidenreich, A., Hofmann, R., Engelmann, U.H. *The use of bisphosphonate for the palliative treatment of painful bone metastasis due to hormone refractory prostate cancer.* J Urol 2001, 165: 136-40.
125. Fernandez-Conde, M., Alcover, J., Aaron, J.E., Ordi, J., Carretero, P. *Skeletal response to clodronate in prostate cancer with bone metastases.* Am J Clin Oncol 1997, 20: 471-6.
126. Dearnaley, D., Sydes, M. *Preliminary evidence that oral clodronate delays symptomatic progression of bone metastases from prostate cancer: First results of the MRC Pr05 Trial.* Am Soc Clin Oncol Annual Meeting 2001.
127. Coleman, R.E. *Optimising treatment of bone metastases by Aredia(TM) and Zometa(TM).* Breast Cancer 2000, 7: 361-9.
128. Boissier, S., Ferreras, M., Peyruchaud, O. et al. *Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases.* Cancer Res 2000, 60: 2949-54.
129. Major, P., Lortholary, A., Hon, J. et al. *Zoledronic acid is superior to pamidronate in the treatment of hypercalcemia of malignancy: A pooled analysis of two randomized, controlled clinical trials.* J Clin Oncol 2001, 19: 558-67.

[CANCER RESEARCH 62, 1832–1837, March 15, 2002]

Use of the Stromal Cell-derived Factor-1/CXCR4 Pathway in Prostate Cancer Metastasis to Bone[1]

Russell S. Taichman,[2] Carlton Cooper, Evan T. Keller, Kenneth J. Pienta, Norton S. Taichman, and Laurie K. McCauley

Departments of Periodontics, Prevention, and Geriatrics, and the Center for Biorestoration of Oral Health, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078 [R. S. T., L. K. M.]; Division of Hematology and Medical Oncology, Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, Michigan 48109 [C. C., K. J. P.]; Department of Pathology and Unit for Laboratory Animal Medicine, School of Medicine, University of Michigan, Ann Arbor, Michigan [E. T. K.]; and Department of Pathology, University of Pennsylvania Dental School, Philadelphia, Pennsylvania 19104 [N. S. T.]

ABSTRACT

Neoplasms have a striking tendency to metastasize or "home" to bone. Hematopoietic cells also home to bone during embryonic development, where evidence points to the chemokine stromal cell-derived factor-1 (SDF-1 or CXCL12; expressed by osteoblasts and endothelial cells) and its receptor (CXCR4) as key elements in these processes. We hypothesized that metastatic prostate carcinomas also use the SDF-1/CXCR4 pathway to localize to the bone. To test this, levels of CXCR4 expression were determined for several human prostate cancer cell lines by reverse transcription-PCR and Western blotting. Positive results were obtained for cell lines derived from malignancies that had spread to bone and marrow. Prostate cancer cells were also observed migrating across bone marrow endothelial cell monolayers in response to SDF-1. In *in vitro* adhesion assays, pretreatment of the prostate cancer cells with SDF-1 significantly increased their adhesion to osteosarcomas and endothelial cell lines in a dose-dependent manner. Invasion of the cancer cell lines through basement membranes was also supported by SDF-1 and inhibited by antibody to CXCR4. Collectively, these results suggest that prostate cancers and perhaps other neoplasms may use the SDF-1/CXCR4 pathway to spread to bone.

INTRODUCTION

Prostate neoplasms have a striking tendency to metastasize to bone. For metastases to occur, the malignant cells must escape the primary tumor, penetrate and circulate through the bloodstream, and subsequently arrest and proliferate in target tissues. The mechanisms that account for bone homing behavior have not yet been elucidated but may include a "direct" vascular pathway, highly permeable marrow sinusoids, chemotactic factors produced by marrow stromal cells, and the synthesis of growth factors by resident cells within the bone and marrow that support the survival, growth, and proliferation of "seeded" cancer cells (1, 2).

It is well known that hematopoietic stem cells also "home" to bone during fetal life and during marrow transplantation (3). In this context, a CXC chemokine SDF-1[3] (or CXCL12) and its receptor, CXCR4 appear to be critical molecular determinants for these events (4, 5). For instance, although normal fetal liver hematopoiesis still occurs in SDF-1 or CXCR4 gene knockouts, marrow engraftment by these hematopoietic cells is not observed (5, 6). In addition, the levels of CXCR4 expression correlate with the ability of human progenitors to engraft into the marrow of nude mice (7). Finally, osteoblasts and marrow endothelial cells express SDF-1 protein that functions as a chemoattractant for human hematopoietic progenitor cells (8–10). Thus, it appears that SDF-1 and CXCR4 represent at least one of the critical determinants for bone marrow homing by hematopoietic cells.

On the basis of the hematopoietic model, we hypothesize that metastatic prostate carcinomas may use a similar pathway to localize to the bone marrow. In the present investigation, we demonstrate that several human prostate cancer cell lines express functional CXCR4 receptors, and that SDF-1 alters the adherence, migration, and invasion of human prostate cancer cell lines. These data are consistent with a role of SDF-1/CXCR4 in metastatic cascades of prostatic carcinomas and suggest novel targets for therapeutic intervention.

MATERIALS AND METHODS

Primary HOB Cells, Osteosarcoma, Endothelial and Prostate Cancer Cell Lines. Enriched HOB cultures were established as detailed previously (11). MG-63 (CRL1424) and SaOS-2 (ATCC 85-HTB) osteosarcoma cell lines were purchased from the ATCC (Rockville, MD). Bone marrow endothelial cells (HBME) were isolated from a normal Caucasian male and immortalized with SV40 large T-antigen (12).

PC-3 and DU145 prostate cancer cells originally isolated from vertebral and brain metastases from prostate cancer patients were obtained from ATCC. LNCaP cells were isolated from a lymph node of a patient with disseminated bony and lymph node involvement (UroCor, Inc., Oklahoma City, OK). The rat MatLyLu cell line were obtained from Dr. John Isaacs (John Hopkins University, Baltimore, MD). MCF-7 cells were established from a patient with metastatic breast cancer (ATCC).

Prostate cancer cell lines were passaged and allowed to grow to confluence over 5 days. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine.

RT-PCR. RT-PCR was performed as described previously (9, 11). Sense and antisense primers were prepared to cross intron/exon boundaries including: SDF-1, 5′-CGT CAG CCG CAT TGC CCG CT and 3′-GGT CTA GCG GAA AGT CCT (380 bp); CXCR4, 5′-GGC AGC AGG TAG CAA AGT GA and 3′-TGA TGA CAA AGA GGA GGT CGG (341 bp); glyceraldehyde-3-phosphate dehydrogenase, 5′-GAC AAC AGC CTC AAG ATC ATC AGC and 3′-AAG TCA GAG GAG ACC ACC TGG TGC; and β-actin, 5′-TCC TGT GGC ATC CAT GAA ACT ACA TTC AAT TCC, 3′-GTG AAA ACG CAG CTC AGT AAC AGT CCG CCT AG (347 bp). The samples underwent thermal cycling at 94°C for 1 min and 60°C for 1 min and 72°C for 1 min for 35 cycles for SDF-1, followed by a 10-min extension at 72°C (Perkin-Elmer, Foster City, CA). PCR for CXCR4 was performed at 94°C, 55°C, and 72°C. False positives and DNA contamination were controlled by omitting reverse transcriptase in control reactions.

Immunohistochemistry. Indirect immunohistochemistry was performed for CXCR4 on cells grown in eight-well tissue slides (Costar Corp). Cells fixed in 2% paraformaldehyde at 25°C for 30 min with Triton X-100 were incubated with either 10 μg/ml of a murine antihuman CXCR4 monoclonal antibody (BD PharMingen, San Diego, CA) in PBS with 10% normal goat serum or an isotype-matched control at 25°C (Sigma Chemical Co., St. Louis, MO), followed by a goat antimurine FITC-conjugated serum at a 1:50 dilution (Sigma Chemical Co.).

SDF-1 ELISA. For determination of SDF-1 levels in conditioned medium, primary human osteoblasts and osteosarcoma cell lines were plated to an initial density of 2.0×10^5 cells/cm^2 in Ham's F-12/DMEM (1:1, v/v) medium

Received 8/20/01; accepted 1/18/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported from a grant from the Specialized Program of Research Excellence in Prostate Cancer at the University of Michigan and NIH Award AR46024.

[2] To whom requests for reprints should be addressed, at Department of Periodontics, Prevention, and Geriatrics, University of Michigan School of Dentistry, 1011 North University Avenue, Ann Arbor, MI 48109-1078. Phone: (734) 764-9952; Fax: (734) 763-5503; E-mail: rtaich@umich.edu.

[3] The abbreviations used are: SDF-1, stromal cell-derived factor 1; HOB, human osteoblast; ATCC, American Type Culture Collection; RT-PCR, reverse transcription-PCR; ERK, extracellular signal-regulated kinase.

containing 10% FBS, antibiotics, 10 mM β-glycerol phosphate, and 10 μg/ml L-ascorbate in 24-well plates (Life Technologies, Inc., Grand Island, NY). Medium was changed on days 3 and 5, then on day 7; after the cells had reached confluence, cells were washed twice in PBS, medium was replaced, and conditioned medium was collected and stored at −80°C. Medium was analyzed by antibody sandwich ELISA (R&D Systems, Minneapolis, MN) with a detection range of 62.5–5000 pg/ml SDF-1.

Western Blot Analysis. Prostate cancer cells were cultured to confluence, and cells were washed and then serum-starved in RPMI with 0.1% BSA for 48 h. SDF-1 stimulation was performed with 0–200 ng/ml SDF-1 in PBS containing 0.1% BSA or vehicle (R&D Systems) for 5–60 min or 24 h. Cells were lysed by freeze-thawing in ice-cold lysis buffer (50 mM Tris-HCl, 1% NP40, 120 mM NaCl, 1 mM EDTA, 25 mM NaF, 40 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 1.0% mammalian protease inhibitor mixture; Sigma Chemical Co.). The nuclei and cellular debris were removed by centrifugation at 16,000 × *g* for 15 min at 4°C. Normalized lysates (30 μg) in Laemmli buffer were electrophoresed on 10% polyacrylamide gels under reducing conditions and transferred to polyvinylidene difluoride membranes. For CXCR4 detection, the membranes were either blocked in 3% BSA in PBS-0.1% Tween 20, and a mouse antihuman monoclonal antibody (1 μg/ml; clone 12G5; PharMingen, San Diego, CA) was used in conjunction with goat antimurine horseradish peroxidase, or membranes were blocked in 5% Blotto in PBS-0.1% Tween 20 and a rabbit anti-CXCR4 antiserum (1:1000 dilution; AB2074; Abcam Corp., Cambridge, United Kingdom) was used in conjunction with goat antirabbit horseradish peroxidase (Sigma Chemical Co.). Final detection was by chemiluminescence (Amersham Pharmacia, Inc., Piscataway, NJ). ERK detection was similarly performed in 5% Blotto with a mouse monoclonal reactive to Tyr-204-phosphorylated ERK1, ERK2, and total ERK1 and ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Cell-Cell Adhesion Assay. Prostate cancer cell lines were labeled with 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4-5-carboxyfluorescein ester (Molecular Bioprobes, Inc., Eugene, Oregon) for 30 min. Labeled cells (1×10^5) were deposited directly onto osteosarcoma MG-63 or SaOS-2 or human bone marrow endothelial cell (labeled as endo) monolayers. SDF-1 pretreatment was performed by incubating the prostate cancer cells with 0–200 ng/ml SDF-1 (or 200 ng/ml SDF-1 that had been boiled for 15 min as a negative control) for 30 min at 37°C. Cell-to-cell adhesion was allowed to proceed for 30 min at 37°C. Adherence was quantified in a 96-well fluorescent plate reader (IDEXX Research Products, Westbrook, ME). Data are presented as raw fluorescent counts.

Transendothelial Migration. HBME cells were seeded onto 12-μM TransWell microporous membrane (Corning Costar Corp., Cambridge, MA) 24-well plates. Prostate cancer cell lines were placed in the upper chamber. Transmigration supported by a SDF-1 gradient (0–200 ng/ml) was achieved by adding various concentrations of SDF-1 in the lower chamber. To evaluate random migration (chemokinesis), SDF-1 was added to both upper and lower chambers. After a 24–30 h incubation, the number of transmigrated cells in the lower chambers were enumerated by direct microscopic counts. Spontaneous transendothelial migration was compared with transmigration supported by a SDF-1 gradient (13).

Invasion of Prostate Cancer Cells. Cell invasion was examined using a reconstituted extracellular matrix membrane (Matrigel, Beckman Coulter Labware, Franklin Lakes, NJ) or type I collagen (Collagen Corp., Palo Alto, CA). Cell invasion chambers were prepared by placing 40 μl of the extracellular matrix into the top chamber of Transwells, which were incubated for 2 h at 37°C. Test cells were placed in the upper chamber (1×10^5 cells/well) in serum-free medium, and 0–400 ng/ml SDF-1 were added to the lower chamber. Spontaneous invasion was compared with invasion supported by a SDF-1 gradient (13). Invasion into the matrix was assayed after 24–30 h by visual quantification of the cells that had migrated into matrix. The effect of 1 μg/ml CXCR4 blocking antibody (12G5 PharMingen) added to the top chamber of the Transwell was used to provide additional verification that observed responses are dependent on CXCR4 receptor binding.

Statistical Analysis. Statistical differences between the means for the different groups were evaluated with Instat 4.0 (GraphPAD software) using one-way ANOVA, with the level of significance at $P < 0.05$. All experiments were repeated two to three times with triplicate samples, and similar results were obtained.

RESULTS

To determine whether SDF-1 and its receptor (CXCR4) help to define the bone specific metastasis of prostate carcinomas, we first identified which elements in the bone marrow express SDF-1 (11, 14). Using RT-PCR, we observed that the majority of the normal primary human osteoblastic cells express mRNA for SDF-1 (Fig. 1*A*), and SDF-1 levels were easily detected in the conditioned medium of four primary osteoblastic cell isolates with levels ranging from 138 ± 36 to 787 ± 48 pg/ml/96 h/10^4 cells (Fig. 1*B*). In addition, several human osteosarcoma cell lines, including MG-63 and HOS TE85 (not presented), also express SDF-1 mRNA, murine bone marrow stromal cells that express an osteoblast phenotype, as well as human osteoblasts *in situ,* express SDF-1 (9) but not the SaOS-2 cell line.

Several human prostate cancer cell lines were evaluated for the expression of the SDF-1 receptor by RT-PCR. Positive controls for CXCR4 expression included RNA collected from hematopoietic (HL-60) and breast cancer (MCF-7) cell lines (15). RNA collected from the SaOS-2 osteosarcoma cell line served as a negative control. Expres-

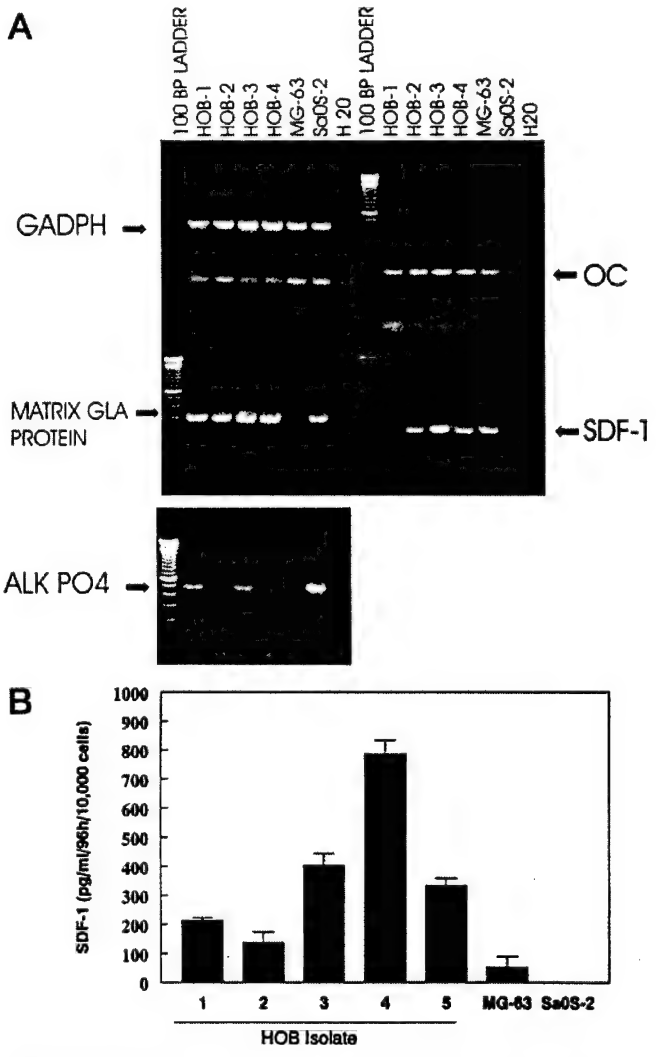

Fig. 1. SDF-1 is expressed by marrow-derived HOBs. In *A*, ethidium bromide-stained gel of RT-PCR performed using RNA obtained from HOBs (*HOB1* to *HOB4*) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), SDF-1, matrix Gla protein, osteocalcin (*OC*), and alkaline phosphatase (*ALK PO4*). In *B*, SDF-1 protein levels were measured in 96-h conditioned medium from five additional HOB cell isolates (*B*). The data indicate that three of four primary HOBs and MG-63 express SDF-1 mRNA and protein, whereas HOB1 and SaOS-2 do not.

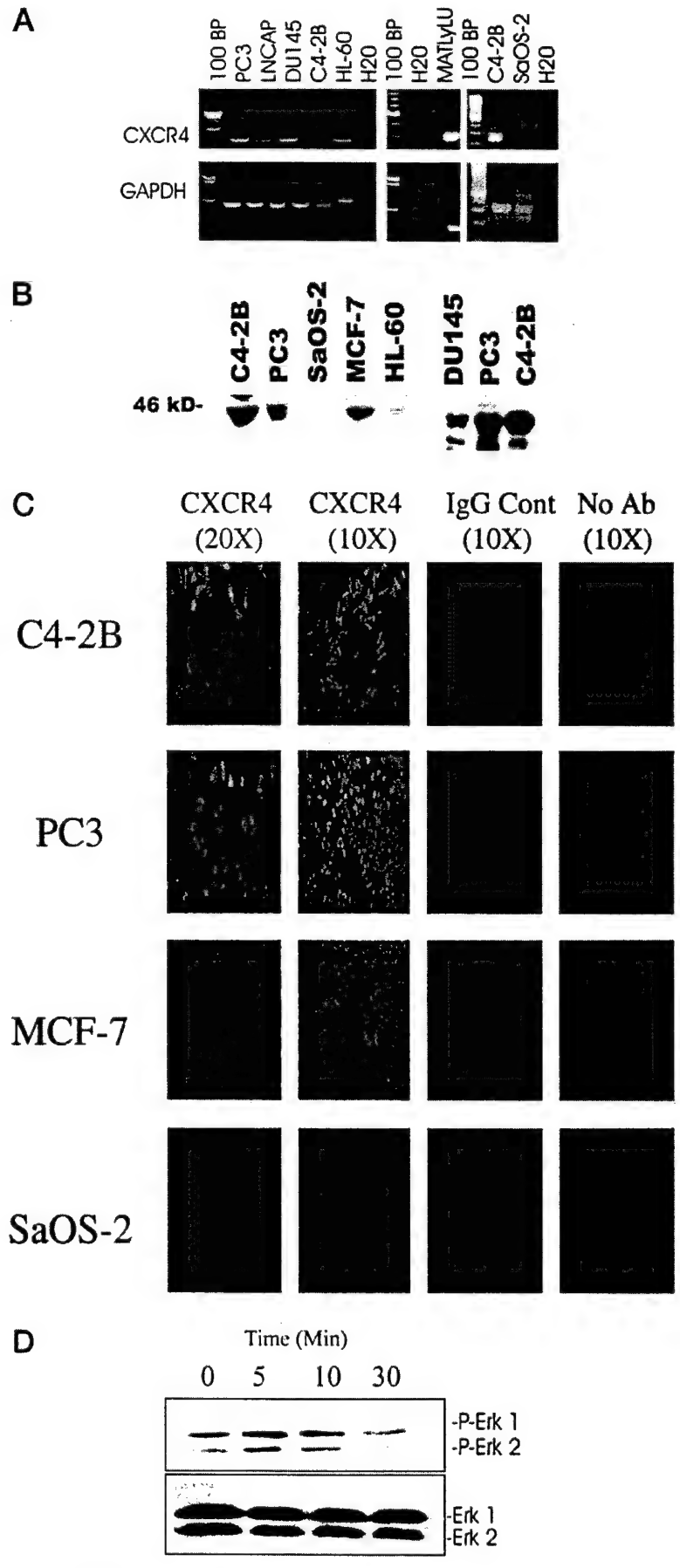

Fig. 2. Functional CXCR4 receptors are expressed by prostate cancer cell lines. In *A*, ethidium bromide-stained gel of RT-PCR performed using RNA recovered from human (DU145, PC3, LNCAP, and C4-2B) and rat (MatLyLu) prostate cancers, with SaOS-2 and HL-60 cells, as negative and positive controls, respectively. CXCR4 mRNA was observed for all cell types evaluated except SaOS-2. *B*, Western blot probed directly with a monoclonal anti-CXCR4 (*B, right*) or a polyclonal antibody (*B, left*), demonstrating a M_r 46,000 band corresponding to the CXCR4 receptor. Controls included protein isolated from MCF-7, HL-60 (positive), and SaOS-2 cells (negative) cells. *C*, immunohistochemistry with an isotype-matched control or CXCR4 antibody demonstrating CXCR4 expression by C4–2B, PC3, and MCF-7 (positive control) but not SaOS-2 (negative control). *D*, Western blots of SDF-1-stimulated PC-3 cells using antibodies that detect total (*bottom*) and phosphorylated (*P*) Erk-1 and Erk-2 proteins (*top*). The data demonstrate that SDF-1 stimulates phosphorylation of ERK proteins in PC3 cells.

sion of CXCR4 was observed in the PC-3 and DU145 cell lines, derived from malignancies that had spread to bone marrow and brain, respectively. Hormone-refractory prostate carcinoma cell lines cloned from a lymph node (LNCaP) and bone marrow (C4–2B) and the rat MatLyLu prostate carcinoma cell line also expressed the CXCR4 mRNA but not the SaOS-2 osteosarcoma cell line (Fig. 2*A*). Confirmation that CXCR4 is expressed by prostate carcinoma cells was obtained using Western blotting using monoclonal and polyclonal antibodies (Fig. 2*B*). The data demonstrate a M_r 46,000 band corresponding to CXCR4 for both MCF-7, HL-60, and the prostate carcinomas but not for the SaOS-2 cell line. Further conformation that prostate cancer cell lines express CXCR4 was obtained using immunohistochemistry to the receptor (Fig. 2*C*).

To verify that the CXCR4 receptors are functional in prostate cancer cell lines, the cells were examined to determine whether ERK-1/ERK-2 pathways are activated upon ligand binding, as has been demonstrated in hematopoietic cells. Rapid activation of phosphorylated ERK-1/ERK-2 proteins was observed within 5 min of SDF-1 stimulation, with ERK-1/ERK-2 levels returning to baseline within 30 min after receptor engagement (Fig. 2*D, top*). No changes in the total Erk protein expression were observed over the course of the investigation (Fig. 2*D, bottom*).

Prostate cancer cells may use CXCR4 receptors to adhere to cells and/or extracellular matrix components in the bone marrow. We carried out experiments to test this possibility by determining whether SDF-1-treated cells adhere preferentially to osteosarcoma cell lines or human bone marrow endothelial cells than untreated controls. SDF-1 pretreatment enhanced the binding of prostate cancer cell lines to human osteosarcoma cells (Fig. 3, *A* and *B*). Similarly, SDF-1 enhanced the binding of several prostate cancer cell lines to human endothelial cells, although the total percentage of bound cells was considerably less (Fig. 3*C*).

Once bound to endothelium, the metastatic tumor cells must migrate across the microvascular barrier to invade and colonize target tissues. As an *in vitro* analogue, we explored whether prostate cancer cells could migrate across endothelial cell monolayers in response to SDF-1. Confluent layers of bone marrow endothelial cells (HBME) were seeded onto Transwell microporous membranes. Migrating cells were introduced into the upper chamber, and a gradient of SDF-1 (0 or 200 ng/ml) was established by placing the chemokine in the lower chamber. Our data support this concept because SDF-1 stimulated the directed movement of the prostate cancer cell lines (Fig. 4).

Once tumor cells have adhered to and moved through the endothelium, they must invade through the extracellular matrix. The ability of SDF-1 and CXCR4 to influence prostate carcinoma invasion were studied using a reconstituted extracellular matrix (Matrigel; Beckman Coulter Labware, Franklin Lakes, NJ) in porous chambers. SDF-1 supported the invasion of PC3 and C4–2B cells into the reconstituted matrix (Fig. 4). Addition of SDF-1 to both chambers of the assay abrogated the invasion. Similar findings were made for the DU145, and PC3 invasion into type I bovine collagen (Collagen Corp.). Serum-free osteoblast conditioned medium also supported invasion, but not medium derived from the SaOS-2 cell line, which is consistent with our RT-PCR data (Fig. 1 and data not presented). Addition of antibody to CXCR4 the top chamber of the culture but not an isotype-matched control significantly reduced the number of invading cells (Fig. 4). As a whole, these data support the role of SDF-1 and CXCR4 in the development of metastasis of prostate carcinomas.

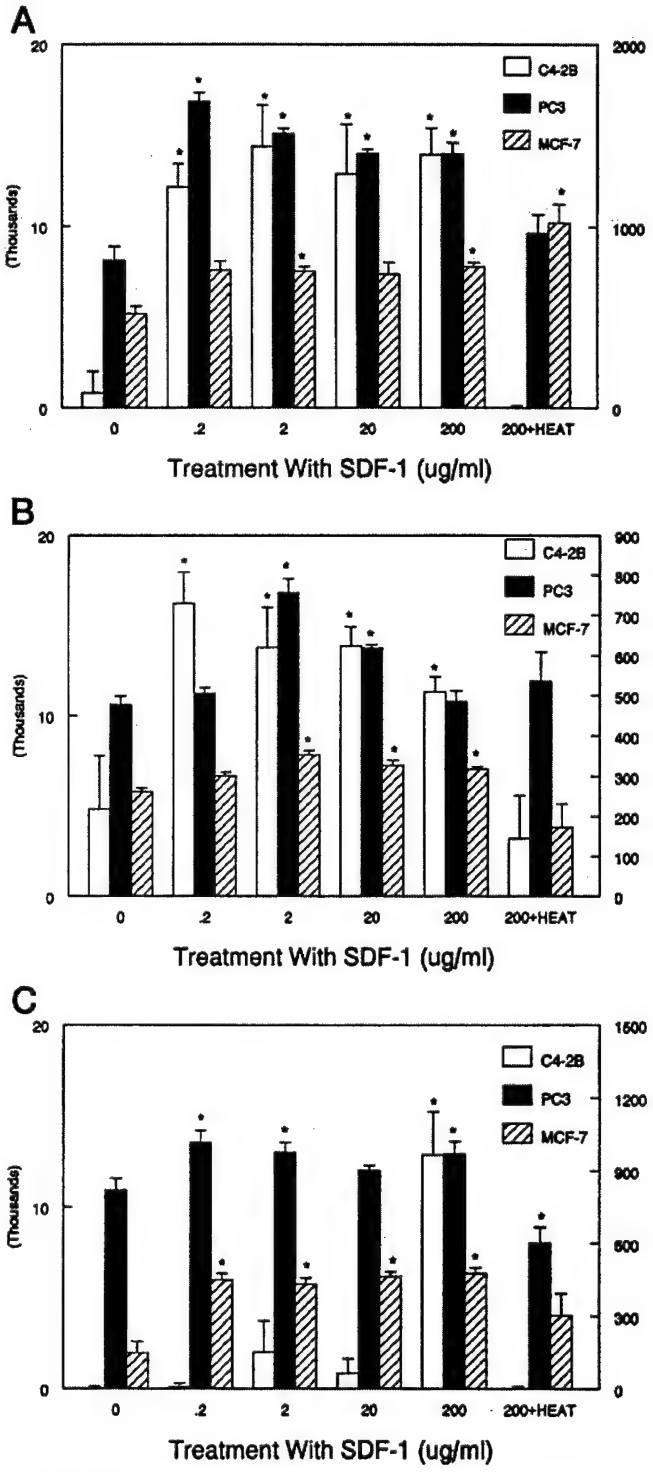

Fig. 3. SDF-1 enhances binding of prostate cancer cells to human osteosarcoma cell lines and human bone marrow endothelium. 3′-*O*-Acetyl-2′,7′-bis(carboxyethyl)-4-5-carboxyfluorescein ester-labeled C4–2B, PC3, or MCF-7 cells (1×10^5) were labeled and deposited directly on to osteosarcoma MG-63 (*A*), SaOS-2 cells (*B*), or human bone marrow endothelial cell (*C*) monolayers. Pretreatment was performed by incubating the prostate cancer cells with 0–200 ng/ml SDF-1 or heat-inactivated 200 ng/ml SDF-1. Cell-to-cell adhesion was allowed to proceed for 30 min at 37°C, and adherence was quantified in a fluorescent plate reader (IDEXX Research Products, Westbrook, ME; $n = 5$). Data for the C4–2B cell line use the right *Y axis*, and PC3 and MCF-7 (positive control) use the *left*, presented as a raw fluorescent counts/well of the mean; *bars*, SD. *, significant from nontreatment control ($P < 0.05$).

DISCUSSION

Prostate cancer is a common neoplasm and the second leading cause of cancer deaths in American males (16). The high mortality

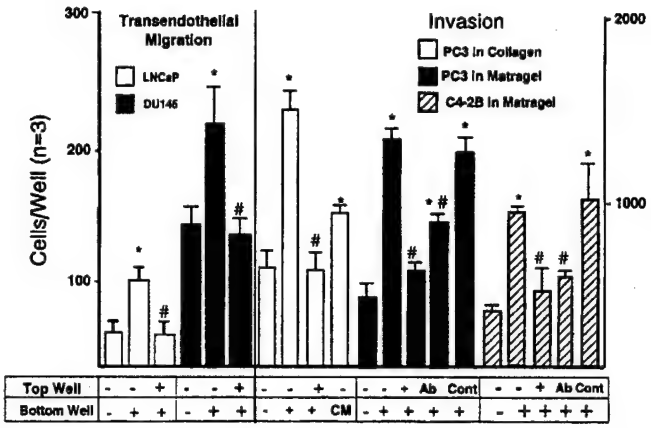

Fig. 4. SDF-1 supports transendothelial migration and invasion of prostate cancer cells. Confluent HBME cells were established in Transwell plates. LNCaP or PC3 cells were suspended in the top well (5×10^4 cells/0.1 ml), and SDF-1 (0 or 200 ng/ml) was placed into the bottom chambers. Chemokinesis was studied by adding SDF-1 to both the upper and lower chambers. Transmigrated cells in the lower chambers were enumerated at 24 h (*left*). Invasion activity (*right*) was examined using a reconstituted extracellular matrix membrane or type I collagen. PC3 or C4–2B cells were placed in the upper chamber in serum-free medium, control (0), or 200 ng/ml SDF-1 (or 50% primary osteoblast conditioned medium collected at 72 h, *CM*) were added to the lower chamber. CXCR4 function-blocking antibody (*Ab*) or isotype-matched control (*Cont*) were used to evaluate specificity. Data are presented as mean migrating cells/well; *bars*, SD ($n = 3$). *, significant difference from nontreatment control; #, significant from invasion or transendothelial migration mediated by SDF-1 (*top*, 0; *bottom*, 200 μg/ml; $P < 0.05$).

rate is principally attributable to the spread of malignant cells to many tissues including bone (17). As a result, there is a growing interest in the early detection and screening of men for prostate cancer and for a greater understanding of the mechanisms that lead to metastasis.

We hypothesized that SDF-1 and its CXCR4 receptor would help to define the bone-specific metastasis of prostate carcinomas. To address this hypothesis, we first identified osteoblasts and endothelial as key elements in the bone marrow that express SDF-1 (9). We observed that primary HOBs produce a wide range of SDF-1 levels. Although we have not attempted to optimize the conditions for SDF-1 synthesis, these data are in keeping with those we reported previously for other cells types including marrow stromal and murine osteoblastic cells (9) and thyroid-derived fibroblasts and adenomas (18) but considerably less than gingival fibroblasts (19). We next examined the expression of CXCR4 in several human prostate cancer cell lines by RT-PCR and by Western blotting and immunohistochemistry. Expression of CXCR4 was observed for PC-3 and DU145 cell lines, derived from malignancies that had spread to bone and brain, respectively. Hormone-refractory prostate carcinoma cell lines cloned from a lymph node (LnCaP) and marrow (C4–2B) also expressed CXCR4.

For prostate cancers to exit the vasculature, they must first adhere to the endothelium and subsequently move through the endothelial monolayer and underlying connective tissues. We observed that in *in vitro* adhesion assays, pretreatment of the prostate cancer cells with SDF-1 significantly increased their adhesion to several osteosarcomas and bone marrow-derived endothelial cells in a dose-dependent manor, suggesting that prostate carcinomas migrate across endothelial cell monolayers in response to SDF-1. Finally, we were able to demonstrate that SDF-1 supported the invasion of prostate carcinoma cell lines into reconstituted basement membranes, and this activity could be blocked by either antibody to the CXCR4 receptor or by using a specific synthetic inhibitor of CXCR4. Collectively, our results suggest this possibility that prostate cancers and perhaps other neoplasms (*i.e.*, breast) use the SDF-1/CXCR4 pathway during their spread to bone and other tissues.

In the bone marrow, SDF-1 is constitutively produced by osteoblasts, fibroblasts, and endothelial cells (9). It is important to point out that vascular endothelial cells in other tissues, such as those lining pulmonary channels, do not secrete SDF-1 (20). Together, selective expression of SDF-1 by endothelial cells and other resident cells in specific tissues may provide a mechanism to localize hematopoietic cells to these tissue compartments. More important than secretion, SDF-1 must be biologically active. SDF-1 is known to bind heparin, heparin sulfated proteoglycans, and fibronectin, which may change the activity of the ligand (21, 22). Indeed, this has been demonstrated recently by Peled *et al.* (23), who showed that heparin-bound SDF-1 was able to arrest $CD34^+$ cells rolling on marrow vascular endothelium. Subsequently, firm adhesions were established by $CD34^+$ cells on endothelium using VLA-4 and LFA-1 receptors, ultimately culminating in the extravasation of $CD34^+$ progenitors into the marrow. As cancer cells also produce humoral factors (including interleukin 1 and tumor necrosis factor) that facilitate the expression of cell adhesion molecules on endothelial cells [*e.g.*, E-selectin, P-selectin, and hyaluronate (ligand for CD44); Refs. 24, 25)], we are currently exploring whether the synthesis of SDF-1 by marrow endothelium and osteoblasts can be altered by prostate carcinoma cells and whether this then further enhances tumor cell adhesions (26, 27).

In addition to chemoattraction, SDF-1 may also help to establish metastases in bone by serving as a growth factor or to prevent apoptosis of the tumor cells. To evaluate this possibility, we cultured for several prostate carcinoma cell lines in serum and serum-free medium in the presence of increasing amounts of SDF-1. SDF-1 alone failed to modulate proliferation of any of the cell lines evaluated (data not presented). It should be noted that SDF-1 does not stimulate proliferation of early hematopoietic cells but synergizes in combination with other growth factors (28). Furthermore, SDF-1 did not the preserve colony formation upon serum starvation, nor did it prevent anoikis of PC3 and C4–2B cells. Thus, although it may be premature to conclude that SDF-1 is without effect on tumor cell growth or survival alone, we have no evidence that SDF-1 in osteoblast or mixed stromal cell-conditioned medium supported prostate cancer cell growth.

Although both blood cells and prostate cancer cells home to bone marrow, we are not aware of any investigation that addresses whether SDF-1/CXCR4 is operating in the pathogenesis of prostate cancer metastasis. This possibility is reinforced by virtue of the fact that the *CXCR4* gene is expressed in normal prostate tissues, albeit at low levels (29). Moreover, both ligand and receptor are overexpressed in several neoplasms that invade the marrow (*e.g.*, breast cancers, Burkitt's lymphoma, several leukemias, and neuroblastomas; Refs. 15, 29–32). On the basis of these considerations, it is reasonable to ask whether CXCR4 receptors are up-regulated in malignant prostate cancer cells, and if they are, do these receptors function to direct malignant prostate cancers to the bone marrow? Moreover, Muller *et al.* (33) recently reported similar findings in a breast cancer model. These authors demonstrated that normal breast tissues express little CXCR4, whereas breast neoplasms express high levels of CXCR4. Furthermore, antibody to CXCR4 blocks the metastatic spread of the tumors to the lung and lymph nodes. Together, these investigations suggest a role of SDF-1/CXCR4 in metastatic cascades of prostate carcinoma and thereby suggest novel targets for therapeutic intervention to prevent prostate cancer metastasis.

ACKNOWLEDGMENTS

We are indebted to Dr. Larry S. Matthews and the members of the Tissue Procurement Core of the University of Michigan for help in obtaining tissues and to Drs. Cun-Yu Wang and David Sarment for helpful discussions.

REFERENCES

1. Geldof, A. A. Models for cancer skeletal metastasis: a reappraisal of Batson's plexus. Anticancer Res., *17:* 1535–1539, 1997.
2. Diel, I. Historical remarks on metastasis and metastatic bone disease. *In:* I. Diel, M. Kaufmann, and Bastert G. (eds.), Metastatic Bone Disease. Fundamental and Clinical Aspects, pp. 1–11. New York: Springer-Verlag, 1994.
3. Hardy, C. Homing of hematopoietic stem cells to the marrow. Am J. Med. Sci., *309:* 260–266, 1995.
4. Kim, C. H., and Broxmeyer, H. E. SLC/exodus2/6Ckine/TCA4 induces chemotaxis of hematopoietic progenitor cells: differential activity of ligands of CCR7, CXCR3, or CXCR4 in chemotaxis *vs.* suppression of progenitor proliferation. J. Leuk. Biol., *66:* 455–461, 1999.
5. Aiuti, A., Tavian, M., Cipponi, A., Ficara, F., Zappone, E., Hoxie, J., Peault, B., and Bordignon, C. Expression of CXCR4, the receptor for stromal cell-derived factor-1 on fetal and adult human lympho-hematopoietic progenitors. Eur. J. Immunol., *29:* 1823–1831, 1999.
6. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature (Lond.), *382:* 635–638, 1996.
7. Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science (Wash. DC), *283:* 845–848, 1999.
8. Aiuti, A., Webb, I. J., Bleul, C., Springer, T., and Gutierrez-Ramos, J. C. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J. Exp. Med., *185:* 111–120, 1997.
9. Ponomaryov, T., Peled, A., Peled, I., Taichman, R. S., Habler, L., Sandbank, J., Arenzana-Seisdedos, G., Nagler, A., Lahav, M., Szyper-Kravitz, M., Zipori, D., and Lapidot, T. Increased production of SDF-1 following treatment with DNA damaging agents: relevance for human stem cell homing and repopulation of NOD/SCID mice. J. Clin. Investig., *106:* 1331–1339, 2000.
10. Hamada, T., Mohle, R., Hesselgesser, J., Hoxie, J., Nachman, R. L., Moore, M. A., and Rafii, S. Transendothelial migration of megakaryocytes in response to stromal cell-derived factor 1 (SDF-1) enhances platelet formation. J. Exp. Med., *188:* 539–548, 1998.
11. Taichman, R. S., and Emerson, S. G. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. J. Exp. Med., *179:* 1677–1682, 1994.
12. Lehr, J. E., and Pienta, K. J. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J. Natl. Cancer Inst. (Bethesda), *90:* 118–123, 1998.
13. Mohle, R., Bautz, F., Rafii, S., Moore, M. A., Brugger, W., and Kanz, L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood, *91:* 4523–4530, 1998.
14. Taichman, R. S., and Emerson, S. G. The role of osteoblasts in the hematopoietic microenvironment. Stem Cells, *16:* 7–15, 1998.
15. Sehgal, A., Ricks, S., Boynton, A. L., Warrick, J., and Murphy, G. P. Molecular characterization of CXCR-4: a potential brain tumor-associated gene. J. Surg. Oncol., *69:* 239–248, 1998.
16. Pienta, K. J., and Esper, P. S. Risk factors for prostate cancer. Ann. Int. Med., *118:* 793–803, 1993.
17. Jacobs, S. C. Spread of prostatic cancer to bone. Urology, *21:* 337–344, 1983.
18. Aust, G., Steinert, M., Kiessling, S., Kamprad, M., and Simchen, C. Reduced expression of stromal-derived factor 1 in autonomous thyroid adenomas and its regulation in thyroid-derived cells. J. Clin. Endocrinol. Metabol., *86:* 3368–3376, 2001.
19. Fedyk, E. R., Jones, D., Critchley, H. O., Phipps, R. P., Blieden, T. M., and Springer, T. A. Expression of stromal-derived factor-1 is decreased by IL-1 and TNF and in dermal wound healing. J. Immunol., *166:* 5749–5754, 2001.
20. Imai, K., Kobayashi, M., Wang, J., Shinobu, N., Yoshida, H., Hamada, J., Shindo, M., Higashino, F., Tanaka, J., Asaka, M., and Hosokawa, M. Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. Br. J. Haematol., *106:* 905–911, 1999.
21. Mbemba, E., Gluckman, J. C., and Gattegno, L. Glycan and glycosaminoglycan binding properties of stromal cell-derived factor (SDF)-1α. Glycobiology, *10:* 21–29, 2000.
22. Sbaa-Ketata, E., Vasse, M., Lenormand, B., Schneider, P., Soria, C., and Vannier, J. P. Fibronectin increases the migration induced by stromal cell-derived factor-1α (SDF-1α) in pre-B acute lymphoblastic leukemia cells. Eur. Cytokine Network, *12:* 223–230, 2001.
23. Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. J. Clin. Investig., *104:* 1199–1211, 1999.
24. Mohamadzadeh, M., DeGrendele, H., Arizpe, H., Estess, P., and Siegelman, M. Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. J. Clin. Investig., *101:* 97–108, 1998.
25. Iademarco, M. F., Barks, J. L., and Dean, D. C. Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF-α in cultured endothelial cells. J. Clin. Investig., *95:* 264–271, 1995.
26. Holzmann, B., Gosslar, U., and Bittner, M. α4 integrins and tumor metastasis. Curr. Top. Microbiol. Immunol., *231:* 125–141, 1998.
27. Coffey, D. S. Do bone marrow endothelial cells have specific house numbers, and how is this involved in metastasis? J. Natl. Cancer Inst., *90:* 84–85, 1998.
28. Hodohara, K., Fujii, N., Yamamoto, N., and Kaushansky, K. Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). Blood, *95:* 769–775, 2000.
29. Gupta, S. K., and Pillarisetti, K. Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. J. Immunol., *163:* 2368–2372, 1999.
30. Mohle, R., Failenschmid, C., Bautz, F., and Kanz, L. Overexpression of the chemokine receptor CXCR4 in B cell chronic lymphocytic leukemia is associated with increased functional response to stromal cell-derived factor-1 (SDF-1). Leukemia (Baltimore), *13:* 1954–1959, 1999.
31. Slanicka, K. M., Nissen, C., Manz, C. Y., Toksoz, S. D., Lyman, and Wodnar-Filipowicz, A. The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow. Exp. Hematol., *26:* 365–373, 1998.
32. Geminder, H., Sagi-Assif, O., Goldberg, L., Meshel, T., Rechavi, G., Witz, I. P., Ben-Baruch, A. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. J. Immunol., *167:* 4747–4757, 2001.
33. Muller, C. A., Homey, B., Sato, H., Ge, N., Catron, D., Buchanan, M., McClanahan, T., Murphy, E., Yuan, W., Wagners, S., Barrera, J., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond.), *410:* 50–56, 2001.

Interleukin-6 and androgen receptor cofactors in prostate cancer xenografts and cell lines. Smith PC, Wallner, L, Keller ET. AACR, 2002.

A variety of growth factors may contribute to the progression of prostate cancer (CaP). Elevation of serum levels of one putative CaP growth factor, interleukin-6 (IL-6), has recently been associated with advanced prostate cancer in patients. Futhermore, IL-6 has been demonstrated to activate the androgen receptor (AR) in the absence of androgen in CaP cell lines. Taken together, these data suggest that IL-6 may contribute to CaP progression through promotion of androgen independence. The goal of the current study was to determine the presence of IL-6 and its receptor components in CaP xenografts (XG). Additionally, we sought to determine if IL-6 influenced the levels of AR co-factors because of its ability to stimulate an androgen response in the absence of androgens. CaP XG were established from either primary tumor or metastases obtained within 2 hours of the patients' death (i.e. rapid autopsy program). Homogenates were made from the XG and subjected to ELISA for determination of IL-6, soluble IL-6 receptor (sIL6R), and gp130 levels. ELISA values were normalized for total protein in the sample. To determine the influence of IL-6 on AR co-factor levels, several CaP cell lines (LNCaP, C4-2B and VCaP) were incubated with IL-6 (10 ng/ml) for 24 h, then total cell extract was subjected to Western analysis for determination of various AR cofactor levels. We evaluated a total of 9 XG from the following sites: prostate (n=1); dura (n=2); lymph node (n=2); sphenoid (n=1); femur (n=1); rib (n=1); liver (n=1). IL-6 was detected in dura (n=1), liver, and both lymph node XG (range: 231- 32,824 pg/pg total protein). sIL-6R was detected in all XG except the prostate and femur (range: 91-281 pg/ng total protein). gp130 was detected in all XG (range: 8.24-1762 pg/ng total protein). Addition of IL-6 to CaP cell lines did not change total levels of the AR cofactors, SRC-1, TIF2, or AIB1. These data demonstrate that IL-6 and its receptor are present in CaP. They also suggest that IL-6 may be expressed in only a subset of metastatic sites, suggesting that it may contribute to target organ specificity. The observation that IL-6 did not alter AR cofactor levels suggests that IL-6 alters association of AR cofactors with the AR (as opposed to increasing cofactors) or that IL-6 activates AR independent of modulating AR cofactors. We conclude that the presence of IL-6 in XG and its previously demonstrated ability to activate AR lend further evidence that it contributes to the progression of CaP.

Anti-Interleukin-6 Monoclonal Antibody Reduces Progression of Androgen Independent Human Prostate Cancer Xenografts in Nude Mice.

Lauren Wallner, Peter C. Smith, Jinlu Dai, Rodney Dunn, Jian Zhang, Zhi Yao, Evan T. Keller. Unit for Laboratory Animal Medicine, and Cancer Center, University of Michigan, Ann Arbor, MI.

AACR 03

Prostate cancer (CaP) is initially androgen dependent and regresses upon androgen deprivation therapy. However, even after an initial response, the cancer eventually reoccurs and continues to grow in the presence of greatly diminished androgen levels. These progressive cancers are termed androgen independent. The mechanism through which androgen independence develops is unknown. Interleukin-6 (IL-6), a pro-inflammatory cytokine, has been linked to CaP including elevated serum levels in men with advanced CaP and the ability of IL-6 to activate the androgen receptor in the absence of androgen. Furthermore, androgens inhibit IL-6 expression, thus upon androgen deprivation, IL-6 levels increase. Taken together, these findings suggest that IL-6 may play an important role in the transition of CaP from an androgen dependent to an independent state. To test this possibility, we inhibited IL-6 activity in orchiectomized mice with established CaP tumors. Specifically, xenografts of the androgen dependent human CaP line LuCAP-35 were established in nude mice. Then mice were orchiectomized and either anti-IL-6 monoclonal antibody (mAb) or isotype-control IgG antibody were immediately administered and continued for 18 weeks. Tumors were measured twice weekly. In the presence of anti-IL-6 mAb, tumor growth rate was diminished whereas the tumor continued to proliferate in mice receiving control antibody. Additionally, administration of anti-IL-6 mAb resulted in a higher survival rate compared to the control group. We conclude that IL-6 is required for the transition from androgen dependent to independent development of LuCaP xenografts in nude mice. This study suggests that inhibiting IL-6 may be a useful adjunct to androgen-deprivation therapy for men with prostate cancer.

P. C. Smith, J.L. Dai, and E.T. Keller. *Unit for Laboratory Animal Medicine, Dept. of Pathology, and Institute of Gerontology, University of Michigan, Ann Arbor, MI, 48109-0940.*

AACR 00

ANTI-INTERLEUKIN-6 ANTIBODY ENHANCES CHEMOTHERAPEUTIC-MEDIATED INHIBITION OF PROSTATE CANCER CELL GROWTH *IN VITRO*.

Advanced prostate cancer (CaP) is resistant to chemotherapeutic interventions. A method to enhance chemotheraputic-mediated killing of CaP would be an important addition to CaP treatment regimens. The cytokine interleukin-6 (IL-6) and its receptor have been identified in CaP tissue by immunohistochemistry. Furthermore, IL-6 promotes CaP cell growth in vitro in an autocrine fashion. Additionally, IL-6 has been demonstrated to inhibit apoptosis in several cell lines. Accordingly, it follows that inhibition of IL-6 may enhance chemotherapeutic killing of CaP cells. To evaluate this, it was first confirmed that three CaP cell lines, DU145, PC3, and LNCaP expressed IL-6. Then, LD25 and LD50 of etoposide and paclitaxcel were determined for each cell line. The cells were then incubated in the presence of anti-human IL-6 antibody (500 ng/ml) or istoype control for 12 hours. Subsequently, either vehicle, or individual drug at LD25 and LD50 was added and the cells incubated for an additional 36 hours. At this point, cell number was determined. For all three cell lines, pre-incubation with anti-IL-6 antibody prior to addition of etoposide or paclitaxcel resulted in lower cell counts (between 10-20%) than cells pre-incubated with istoype control antibody followed by addition of the chemotherapeutic agents. This effect was observed at both the LD25 and LD50 for each drug. These results show that anti-IL-6 antibody enhances chemotherapeutic mediated inhibition of CaP cell growth in vitro. This finding provides the rationale for evaluation of anti-IL-6 antibody as an adjuvant for CaP chemotherapy in animal models.